THE BIOCHEMICAL JOURNAL

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THE BIOCHEMICAL JOURNAL

SUGGESTIONS TO AUTHORS
ABBREVIATIONS AND SYMBOLS
AND
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REVISED 1953

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ABBREVIATIONS AND SYMBOLS

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PAPERS submitted for publication in the *Biochemical Journal* should be written concisely. Special attention is directed to the sections below concerning the preparation of the typescript. Strict observance of these requirements will shorten the interval between the receipt of a paper and its publication. Typescripts which are not concise or which do not conform to the conventions of the *Biochemical Journal* will be returned to authors for revision.

The attention of authors is drawn to a pamphlet entitled *General Notes on the Preparation of Scientific Papers* published for the Royal Society by the Cambridge University Press (2*s*. 6*d*., post free 2*s*. 9*d*.).

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SUGGESTIONS TO AUTHORS

General. Submission of a paper to the Editorial Board will be held to imply that it presents the results of original research not previously published, that it is not under consideration for publication elsewhere, and that if accepted for the *Biochemical Journal* it will not be published otherwise in the same form, either in English or in any other language, without the consent of the Editorial Board.

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Papers intended for publication should be in double-spaced typing on sheets of uniform size with wide margins. Top copies only should be submitted. The paper should be written in English.

Generally speaking, papers should be divided clearly into parts and, in most cases, these should be as follows: (*a*) Introduction, containing the reasons for doing the work; (*b*) Experimental methods: with chemical papers the experimental part will normally appear towards the end, but otherwise should follow the introduction; (*c*) Results; these should be given concisely: tables or figures are often the best form, but the use of both to illustrate the same data will only rarely be permitted; illustrative protocols only should be included; (*d*) Discussion: it is desirable that the presentation of the results and the discussion of their significance should be considered separately; (*e*) Summary: a summary, about 3 % of the length of the paper, should be included; the paragraphs of the summary should be numbered; (*f*) Acknowledgements; (*g*) References.

The *Biochemical Journal* uses as a standard for spelling the *Concise Oxford Dictionary of Current English* (1951, Oxford: Clarendon Press).

References. These should be given in the text thus: Barnett & Robinson (1942), (Culbertson & Thomas, 1933); where a paper to be cited has more than two authors, the names of all the authors should be given when reference is first made, e.g. (Osborne, Mendel & Ferry, 1919); subsequent citations should appear thus: (Osborne *et al.* 1919). Where more than one paper by the same authors has appeared in one year the reference should be given as follows: Osborne & Mendel (1914*a*); Osborne & Mendel (1914*b*); or Osborne & Mendel (1914*a*, *b*); (Osborne & Mendel, 1914*a*, 1916; Barnett & Robinson, 1942).

At the end of the paper references should be given in alphabetical order according to the name of the first author of the publication quoted, and should include the authors' initials, but not the title of the paper. Titles of journals should be abbreviated in accordance with the system used in the *World List of Scientific Periodicals* (1952, 3rd ed., London: Butterworth). Examples of such abbreviations will be found in the current numbers of the *Biochemical Journal*. References to books and monographs should include the town of publication and the name of the publisher, as well as the date of publication and the number of the edition to which reference is made.

Statistical Treatment of Data. In general it is not necessary to publish the individual results of a number of similar experiments. A statement of the number of individual results, their mean value, the standard error of the mean, and the extreme range of values is usually sufficient. A convenient form in tables is:

$263 \pm 2{\cdot}5$	mean ± S.E.
(10)	no. of results
257–268	range

Alternatively, it is often better, if possible, to include a brief frequency distribution.

A statement that a significant difference exists between the mean (or other) values of two groups of data should be accompanied by the probability derived from the test of significance applied.

Illustrations. Illustrations, which should be approximately twice the size of the finished block, should each be on a separate sheet and packed flat; they should bear the author's name and the title of the paper on the back. Diagrams should be in indian ink and should be drawn on plain white paper, Bristol board, faintly blue-lined graph paper, tracing paper or tracing cloth. Curves based on experimental data should carry clear indications of the experimentally determined points. Letters, numbers, etc., should be written lightly in pencil. Legends and captions should be typed on a separate sheet from the illustrations and numbered correspondingly. Figures should be comprehensible without reference to the text.

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Tables. Tables should carry headings describing their content and should be comprehensible without reference to the text. The units in which the results are expressed, e.g. g./100 ml., should be given at the top of each column, and not repeated on each line of the table.

The following, from *The Presentation of Papers for the Journal of the Chemical Society*, 1952, may be noted: 'Care is needed for statement of powers in column headings. An entry "2" in a column headed "10^3k" means that the observed value was 0·002. If the heading reads "$10^{-3}k$", then the observed value was 2000. This form, 10^xk, should always be used.'

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Chemical Formulae. These should be written as far as possible on a single horizontal line. With inorganic substances and $CHCl_3$, CCl_4 and CS_2, formulae may be used in the text as abbreviations, particularly in the experimental portion, at the discretion of the editors. With salts it must be stated whether or not the anhydrous material is used, e.g. anhydrous $CuSO_4$, or which of the different crystalline forms is indicated, e.g. $CuSO_4$, $5H_2O$; $CuSO_4$, H_2O. (See also p. 16.)

Moles. The word 'mole' is used with the meaning of 'gram-molecule'. It must not be abbreviated to 'mol.' The sub-multiples should be printed as 'm-mole' and 'μmole'.

The number of molecular proportions of reagents employed in a reaction, often referred to in everyday language as 'molecules', may be abbreviated in Experimental sections to 'mol.prop.'.

Description of Solutions. Solutions of common acids and bases should always be expressed in terms of normality (N), and salts preferably in terms of molarity (M), e.g. N-HCl; 0·1 M-NaH_2PO_4. Fractional concentrations should preferably be expressed in the decimal system, e.g. 0·25 N-HCl (not N/4 HCl). The term '%' must be used in its correct sense, i.e. g./100 g. of solution. For 'per cent by volume', i.e. ml./100 ml., the term '% (v/v)' may be employed. To indicate that a given weight of substance is contained in 100 ml. of solution, the term '% (w/v)' (weight per volume) may be used.

Chemical Nomenclature. In the matter of chemical nomenclature the system of the Chemical Society is generally followed. The fundamental principles of organic chemical nomenclature are given by the Definitive Report of the Organic Commission of I.U.P.A.C. (see *J. chem. Soc.*, 1952, p. 5064). The most comprehensive account is given by Mitchell in *British Chemical Nomenclature* (1948, London: Arnold), but this is supplemented and in parts superseded by the Editorial Reports on Nomenclature in *J. chem. Soc.* 1950, p. 3699; 1951, p. 3516; 1952, p. 5057. (See also p. 16.)

Spectrophotometric terms and symbols are those proposed by the Society of Public Analysts and other Analytical Chemists (see *Analyst*, 1942, **67**, 164).

Nomenclature of Micro-organisms. Binominal Latin names of micro-organisms, the generic name only with a capital, must be used in accordance with the International Rules of Nomenclature. Binominals should be underlined once (for *italic*) in the typescript. A name must be given in full at the first mention in a paper; in subsequent mention the generic name may be abbreviated, but the abbreviation must be unambiguous. Trivial names are not underlined and should be without capitals.

Microfungi should be designated as in Ainsworth and Bisby's *A Dictionary of the Fungi* (1950, 3rd ed., Kew: Imperial Mycological Institute).

For bacteria the Editorial Board prefers that the nomenclature of Bergey's *Manual of Determinative Bacteriology* (1948, 6th ed., London: Baillière, Tindall and Cox) should be followed, except in so far as it is in conflict with decisions of the International Committee on Bacteriological Nomenclature. Where authors wish, for good reasons, to use a name other than one approved by the International Committee, the approved name should be inserted in brackets at the first citation.

ABBREVIATIONS AND SYMBOLS

INTRODUCTION

General. The following list gives abbreviations which may be used in the text of papers and symbols which are essentially for use in mathematical formulae but may also be used in the text.

In the Introduction, Results, Discussion and Summary sections of papers the use of abbreviations should be very sparing. Thus, abbreviations for units are to be used, but not contractions such as 'soln.' or 'recryst.' or chemical symbols as abbreviations for elements, groups or compounds, unless this use is justified by special circumstances.

The abbreviation for the plural is the same as that for the singular, unless confusion is likely to arise. Thus 'centimetres' is 'cm.' *not* 'cms.'

Chemical Formulae and Abbreviations. Elements, groups, inorganic compounds (except water), and chloroform, carbon tetrachloride and carbon disulphide are to be denoted by symbols in the Experimental portion of the paper, but not generally in other portions. Diatomic molecules are to be denoted appropriately, e.g. H_2, O_2, N_2, I_2.

In the list which follows the abbreviations marked † for certain compounds may be used, but the full name (with the abbreviation in parentheses) must be given the first time the compound is mentioned in each paper. Among the abbreviations marked † is a set of abbreviations for the names of amino acids and their residues taken from a review by Brand & Edsall (1947, *Annu. Rev. Biochem.* **16**, 224). These abbreviations are to be used only to represent the structures of polypeptides (e.g. Gly.Ala = glycylalanine), and the formulae of proteins (cf. the example given by Brand & Edsall) and, in tables, for amino acids. Similar abbreviations will be used for monosaccharide residues (cf. *J. chem. Soc.* 1952, p. 5121). A key to the abbreviations must be given at the first mention in each paper. The abbreviations GSH and GSSG are used for reduced and oxidized glutathione. DPN and TPN may be used in the text as abbreviations for diphosphopyridine nucleotide and triphosphopyridine nucleotide, respectively; the oxidized and reduced forms, where these must be specified, will be referred to as oxidized DPN, reduced DPN, etc. If DPN and TPN are used as symbols in equations, then the state of oxidation or reduction may be indicated by any logical system, which must be explained in the text.

In chemical names elements carrying substituents are shown by symbols in italics, e.g. *N*-methyl-, used, if necessary, with primes, superscript numerals or Greek letters, e.g. N^4-acetylsulphanilamide. The following forms are used: C_{20} acid, an acid containing 20 C atoms; C-3 or $C_{(3)}$, the atom numbered 3; *C*-Me, carbon-methyl group.

Isotopes. The atomic weight is indicated by superscript figures *before* the symbol of the element, e.g. ^{14}C, ^{15}N. The representation of labelled compounds is referred to on p. 16.

Symbols. In general the *Biochemical Journal* follows the '*Report by the Symbols Committee of the Royal Society on Symbols, Signs, and Abbreviations recommended for British Scientific Publications*' (1951) in the edition published by the Chemical Society and with the reservations that (*a*) full points will be used after abbreviations in lower-case letters for all units, (*b*) temperature on the centigrade scale is shown simply as °, not ° C, (*c*) the abbreviations 'hr.' and 'sec.' continue to be used, and (*d*) μm and μμ are preferred to n and p as prefixes indicating $\times 10^{-9}$ and $\times 10^{-12}$. The following list includes some of the more frequently used symbols, and certain special symbols not included in that report.

Symbols for purely mathematical constants and operators are printed in roman type, e.g. e, base of natural logarithms. Other symbols for quantities, as distinct from abbreviations, are printed in italic type, when they are not Greek, e.g. *d* (density); *E* (extinction or electrode potential); *k*, *K* (velocity and equilibrium constants). They are not followed by a full point except at the end of a sentence. Abbreviations for units which are based on proper names (e.g. ampere, volt) will in future be designated by small capitals, e.g. A, V.

Micro-organisms. The abbreviations marked * for generic names of micro-organisms may be used, but the full name must be given the first time the organism is mentioned in each paper.

LIST OF ABBREVIATIONS AND SYMBOLS

absolute	abs.
acceleration due to gravity	*g*
acetyl, $CH_3.CO$ (in formulae)	Ac
†adenosine diphosphoric acid	ADP
†adenosine triphosphoric acid	ATP
**Aerobacter*	*Aero.*
†alanine	Ala
alkyl group (in formulae)	Alk
alpha-	α-
alternating current	a.c.
ampere	A
analytical standard of purity	A.R.
Ångström unit	Å
approximately	approx. (*or use* about, *not c.* or *ca.*)
†arabinose	Ara
†arginine	Arg
aryl group (in formulae)	Ar
†asparagine	($Asp.NH_2$)
†aspartic acid	Asp
**Aspergillus*	*A.*
atmosphere	atm.
atomic weight	at.wt.
**Azotobacter*	*Az.*
**Bacillus*	*B.*
**Bacterium*	*Bact.*
benzoyl, $C_6H_5.CO$ (in formulae)	Bz
boiling point	b.p., *pl.* b.p.'s
†British anti-lewisite (2:3-dimercaptopropanol)	BAL
British Pharmacopoeia	B.P. (with date)
British Thermal Unit	B.Th.U.
calculated	calc.
Calorie (large, kilogram-calorie)	kcal.
calorie (small, gram-calorie)	cal.
candle power	c.p.
centi- (prefix, 10^{-2})	c
centimetre	cm.
centimetre gram second	c.g.s.
cerebrospinal fluid	c.s.f.
**Chromobacterium*	*Chromobact.*
**Clostridium*	*Cl.*
coefficient	coeff.
compare	cf.
concentrated	conc.
concentration	concn.
concentration (symbol, e.g. in specific rotation)	*c*
configuration (amino acids and carbohydrates and other compounds correlated with glyceraldehyde) (*Biochem. J.* 1948, **42**, 1; 1952, **52**, 1) (for the optical activity of compounds of unknown configuration see under dextrorotatory, laevorotatory)	D-, L-, etc.
constant	const.
constant, equilibrium	*K*
constant, velocity	*k*
corrected (e.g. m.p., for emergent stem)	corr.
**Corynebacterium*	*C.*
crystalline, crystallized	cryst.
cubic	cu. *or, as* e.g. $mm.^3$
curie	C
cycles per second	cyc./sec.
†cysteic acid	($Cy.SO_3H$)
†cysteine	(CySH)
†cystine	(Cy_2S_2)
decomposition (m.p.)	decomp.
degrees absolute (Kelvin)	°K
‡degrees Centigrade	°C

*† See introduction, p. 5.

‡ Centigrade scale assumed in absence of indication; c to be inserted only when more than one scale is used.

degrees Fahrenheit	°F
density	*d*
†deoxyribose	deoxyRib
dextrorotatory (as prefix) (see also 'configuration')	*dextro*, (+)-
diffusion constant	*D*
†3:4-dihydroxyphenylalanine	dopa
dilute	dil.
†diphosphopyridine nucleotide	DPN
direct current	d.c.
dissociation constant, minus log. of	p*K*
electrode potential	*E*
electrode potential, standard	E_0
electrode potential, standard at constant pH	E'_0
electromotive force	e.m.f.
electron volt	ev
equation	eqn.
equivalent (weight)	equiv.
**Escherichia*	*Esch.*
ethyl, C_2H_5 (in formulae)	Et
Experiment (with reference numeral)	Expt.
extinction ($\log_{10} I_0/I$) ($E^{1\%}_{1\,cm.} = E/cl$, where c is concn. (% w/v) and l is length in cm.)	*E*
extinction, molecular (E/cl, where c is molar concn. and l is length in cm.)	ϵ
farad	F
feet per second	ft./sec.
Figure (with reference numeral)	Fig.
†flavin-adenine dinucleotide	FAD
foot	ft.
foot candle	ft.c.
freezing point	f.p.
†fructose	Fru
†galactose	Gal
gallon (Imperial)	gal.
gas constant per mole	***R***
†glucose	G
†glutamic acid	Glu
†glutamine	($Glu.NH_2$)
†glutathione, oxidized	GSSG
†glutathione, reduced	GSH
†glycine	Gly
gram	g.
gram-calorie	cal.
gram-ion	g.ion
gram-molecule	g.mol. (*or* mole)
Gram-negative	Gram −
Gram-positive	Gram +
gravity, acceleration due to	***g***
†haemoglobin	Hb
**Haemophilus*	*H.*
half-wave potential (polarography) (cf. *Biochem. J.* **42**, 421)	*E*
height	ht.
henry	H
high frequency	h.f.
†histidine	His
horse power	h.p.
hour	hr.
hydrogen-ion concentration	$[H^+]$
hydrogen-ion concentration, minus log. of	pH, *pl.* pH's
hydrogen pressure (in atmospheres) in equilibrium with oxidation-reduction system, minus log. of	rH
†hydroxylysine	Hylys
†hydroxyproline	Hypro
inactive (optically)	(±)-
inch	in.
infrared	i.r.
insoluble	insol.

*† See introduction, p. 5.

international unit	i.u.
ionic strength	*I*
*isoleucine	Ileu
joule	J
junior	jun.
kilo- (prefix, $10^3\times$)	k
kilovolt-ampere	kVA
kilowatt-hour	kwh
**Lactobacillus*	*Lb.*
laevorotatory (as prefix) (see also 'configuration')	laevo, (−)-
†leucine	Leu
**Leuconostoc*	*Ln.*
litre	l.
logarithm, in text	log.
logarithm (base 10), in formulae	$\log_{10}$
logarithm (base e), in formulae	ln
low frequency	l.f.
†lysine	Lys
†mannose	Man
maximum	max.
median effective dose	ED_{50}
median lethal dose	LD_{50}
mega- (prefix, $10^6\times$)	M
melting point	m.p., *pl.* m.p.'s
meta (in names of organic compounds)	*m-*
metabolic quotient of X (in μl. X/mg. dry wt. of biological material/hr.)	Q_x‡
metabolic quotient of X (in any other units)	q_x‡
†methionine	Met
methyl, CH_3 (in formulae)	Me
metre	m.
micro- (prefix, $10^{-6}\times$)	μ
microgram	μg. (not γ)
micromicro- (prefix, $10^{-12}\times$)	μμ (*or* p)
micromicron (10^{-12} metre)	μμ.
micromilli- (prefix, $10^{-9}\times$)	μm (*or* n)
micron (10^{-6} metre)	μ.
milli- (prefix, $10^{-3}\times$)	m
milli-equivalent	m-equiv.
millilitre	ml.
millimicron (10^{-9} metre)	mμ.
§millimolar (concentration)	mM
minimum, minute	min.
§molar (concentration)	M
molecule, molecular	mol.
molecular extinction coefficient $=E/cl$, where c is molar concn. and l is length in cm.	ε
molecular optical rotation ($[M]=[\alpha]_D\times$ mol.wt./100)	$[M]_D^{20}$, etc.
molecular proportion (in reactions)	mol.prop.
molecular weight	mol.wt.
**Mucor*	*M.*
**Mycobacterium*	*Myco.*
**Neisseria*	*N.*
non-protein nitrogen (with definition)	N.P.N.
§normal (concentration)	N
normal (in names of organic compounds)	*n-*
normal temperature and pressure (*see* standard temperature and pressure)	
number (in enumerations)	no.
observed	obs.
ohm	Ω
ortho (in names of organic compounds)	*o-*

*† See introduction, p. 5.

‡ See notes on p. 15.

§ Separated by a hyphen (and no full stop) from a chemical formula following it, e.g. M-NaCl; N-NaOH; but M solution.

page, pages	p., pp.
para (in names of organic compounds)	*p*-
parts per million	p.p.m.
**Pasteurella*	*Past.*
**Penicillium*	*P.*
per (e.g. grams per litre)	/ (e.g. g./l.)
‡per cent	%
population parameter corresponding to the S.D. of a sample. (Not to be used except where reference is made to the population parameters)	σ
phenyl, C_6H_5 (in formulae)	Ph
†phenylalanine	Phe
potential difference	p.d.
precipitate	ppt.
preparation	prep.
probability of an event being due to chance alone (i.e. low values imply significant main effect)	*P*
†proline	Pro
**Propionibacterium*	*Pr.*
**Proteus*	*P.*
**Pseudomonas*	*Ps.*
reciprocal centimetre	$cm.^{-1}$
recrystallized	recryst.
red blood corpuscles	R.B.C.
refractive index	n (with wavelength as subscript and temperature as superscript)
relative band speed (partition chromatography). Movement of band/movement of liquid surface above column (Martin & Synge, 1941, *Biochem. J.* **35**, 1358)	*R*
relative band speed (paper-partition chromatography). Movement of band/movement of advancing front of liquid (Consden, Gordon & Martin, 1944, *Biochem. J.* **38**, 224)	R_F
relative humidity	r.h.
respiratory quotient	R.Q.
revolutions	rev.
**Rhodospirillum*	*Rsp.*
röntgen unit	R
**Saccharomyces*	*S.*
**Salmonella*	*Salm.*
second (time)	sec.
secondary (in names of organic compounds)	*sec.*-
sedimentation constant	*S*
†serine	Ser
**Shigella*	*Sh.*
soluble	sol.
solution	soln.
species (sing. and pl.)	sp., spp.
specific gravity	sp.gr.
specific heat	sp.ht.
specific optical rotation (with c in g./100 ml.)	$[\alpha]_D^{20}$, $[\alpha]_D$, $[\alpha]_{5461}^{t}$, etc.
**Spirillum*	*Sp.*
square	sq. *or, as* e.g. $cm.^2$
standard deviation	S.D.
standard error	S.E.
standard error of the mean	S.E.M.
standard temperature and pressure	s.t.p.
**Staphylococcus*	*Staph.*
**Streptococcus*	*Strep.*
substituents (variable, in organic compounds)	R, R′
sum	Σ

*† See introduction, p. 5.

‡ Percentage should be written in full, except in the Methods parts of papers and in Tables, where the symbol '%' may be used for the noun.

temperature	temp.
tertiary (in names of organic compounds)	*tert.*-
†threonine	Thr
time	*t*
†trichloroacetic acid	TCA
†1:1:1-trichloro-2:2-di-(*p*-chlorophenyl)ethane	DDT
triphosphopyridine nucleotide	TPN
†tryptophan	Try
†tyrosine	Tyr
ultraviolet	u.v.
uncorrected (m.p., for emergent stem)	uncorr.
†valine	Val
value (e.g. iodine value)	val.
vapour density	v.d.
vapour pressure	v.p.
variety (e.g. botanical)	var.
velocity	*v*
**Vibrio*	*V.*
viscosity	η
volt	v
volume	vol.
watt	w
wavelength	λ
wavelength of *D* line of sodium (other wavelengths in A)	D (as subscript)
wave number (unit)	$cm.^{-1}$
wave number (symbol)	ν
weight	wt.
†xylose	Xyl
yard	yd.

*† See introduction, p. 5.

NOTES ON USAGE AND CONVENTIONS

SPELLINGS, ETC., ADOPTED

Adopted form	*Remarks*
acetamido	*not* acetylamino
acetyl-β-methylcholine	at first mention give systematic name, β-acetoxypropyltrimethylammonium chloride
adenosine diphosphoric acid	see abbreviations
adenosine triphosphoric acid	see abbreviations; the three phosphorus atoms are distinguished as α, β and γ, thus: adenosine—P^{α}—O—P^{β}—O—P^{γ}
adrenaline	
aetio (prefix)	roman, *no* hyphen
albumin	
(aliquot: *not used*)	substitute 'sample' or 'portion'
alkali, *pl.* alkalis	
allo	italic, *no* hyphen
amino acid	no hyphen
ampoule	
analyse	others words from root $\lambda\acute{\upsilon}\omega$ end in 'lyse'
(aneurin: *not used*)	*use* thiamine, *alternative permitted* vitamin B_1

anti-	hyphenate before compound words (e.g. anti-blacktongue), but join with others (e.g. antiserum, antistreptococcal)
artifact	
ascorbic acid	*alternative permitted* vitamin C
baker's yeast	
benzamido	*not* benzoylamino
benzene (C_6H_6)	*not* benzol; benzine *not used*
benzyloxycarbonyl ($C_6H_5.CH_2.O.CO$—)	*not* carbobenzoxy *or* carbobenzyloxy
bis	roman
bivalent*	
body weight	
boiling point	*no* hyphen
brei	Anglicized, *pl.* breis
brewer's yeast	
Büchner	
(calciferol)	*use* ergocalciferol, *alternative permitted* vitamin D_2
(carbobenzyloxy)	*use* benzyloxycarbonyl
cellophan	
choline	
cholinesterase	
cis-	italic, hyphen
cocarboxylase	
Congo red	
connexion	
cyclo	italic, *no* hyphen
cytochrome *c*	
deoxy (prefix)	*not* desoxy
diiodotyrosine	
dioxan	
disulphide group	*alternative permitted* S.S group
dithionite	preferably $Na_2S_2O_4$; previously called hydrosulphite, hyposulphite
egg white	
electron micrograph	*not* electron microgram
end point	
enzymic	*not* enzymatic
ethanol, ethanolic	*not* ethyl alcohol, *not* alcoholic

* Latin prefixes will be used with words of Latin origin such as 'molecular' and '-valent', e.g. 'unimolecular', 'bivalent' 'ter-', 'quadri-', 'quinque-', 'sexa-', etc.; *not* 'monomolecular', 'divalent', 'tri-', 'tetra-', 'penta-', 'hexa-', etc.

filter paper

flavin-adenine dinucleotide

fluorimetry . . . . . measurement of fluorescence; *not* fluorometry

focusing

fractions, e.g. one-half, two-thirds . . *use* hyphen

freezing point

gelatin

glycerophosphoric acid . . . . . *alternative permitted* phosphoglycerol

guinea pig

haem, protohaem . . . . . prosthetic group of haemoglobin

haematin, protohaematin . . . . . oxidized haem

haemochromogen . . . . . haem + base

haemoglobinometry

hexose phosphoric acid . . . . . three words

(homogenate: *not used*) . . . . . *alternative permitted* any term not implying homogeneity

hydrolysate

hydrolyse

(hydrosulphite, hyposulphite: *not used*) . see dithionite

hydroxyl group . . . . . *alternative permitted* OH group; *not* hydroxy group

ice water

infrared . . . . . one word, *no* hyphen

iodometric

ions:
antimony, quinquevalent, Sb^{5+}
antimony, tervalent, Sb^{3+}
calcium, Ca^{2+}
sulphate, SO_4^{2-}

-ise, -ize . . . . . *use* termination 'ize' generally, but not 'lyze' for words ending in '-lyse'. See Collins, *Authors' and Printers' Dictionary* (1946, 9th ed., Oxford University Press)

*iso*butanol . . . . . *iso*Butanol at the beginning of a sentence

isoelectric

isoleucine

kephalin

light petroleum . . . . . *not* petroleum ether

lipid

litter mate

lyse . . . . . spell with 's' not 'z'; derived words analogously

melting point	
Messrs XYZ Ltd.	no comma before 'Ltd.' 'Messrs' used only in acknowledgements
methanol, methanolic	*not* methyl alcohol
micro-analysis microcrystalline micro-Kjeldahl micro-organism	general rule: no hyphen except before vowel or before proper name
mustard gas	di-2-chloroethyl sulphide *preferred*
ninhydrin	
non-	hyphen
nor (in organic compounds)	roman, *not* italic, *no* hyphen
ox	domestic bovine species without sexual distinction; *not* beef
phenyl glucuronide	two words: for formal nomenclature see *J. chem. Soc.* 1952, p. 5117
phenyl sulphuric acid	*not* phenol sulphate
phlorrhizin	
phospholipid	*not* phospholipin
photomicrograph	
planchet	
potassium hydroxide	*not* caustic potash
pyridoxine, pyridoxal	*alternative permitted* vitamin B_6 (see *Biochem. J.* **52**, 1)
pyrrole	
quadrivalent	
quinol	*not* hydroquinone
quinquevalent	
riboflavin	*alternative permitted* vitamin B_2
semi-	hyphen, except in chemical names, e.g. semicarbazide
separating funnel	
sexavalent	
sodium hydroxide	*not* caustic soda
solvent systems	*use* colons, e.g. butanol:acetic acid:water (40:10:10, v/v)
stilboestrol (4:4′-dihydroxy-$\alpha\beta$-diethylstilbene)	given systematic name first time; *not* called diethylstilboestrol
Student's *t* test	no literature reference
sulphydryl group	*alternative permitted* SH *or* thiol group

tap water	
tervalent	
thiamine	*alternative permitted* vitamin B_1
thiophen	*no* final e
thyroxine	
tocopherol	*alternative permitted* vitamin E
Tollens reagent	
toluene	*not* toluol
trans-	italic, hyphen
trichloroacetic acid	see abbreviations
triose phosphate	
tryptophan	other amino acids have terminal 'e'
twofold	for numbers above ten thus, 11-fold
ultraviolet	one word; *no* hyphen
un-ionized	
univalent	
water	*not* H_2O
water bath	
wavelength	
X-ray	
χ^2 test	

ANALYSES AND PHYSICAL PROPERTIES

The standard forms for reporting these are shown in the following examples:

The *new compound* (name in italics) had m.p. 175° (decomp.); $[\alpha]_D^{22} + 17° \pm 2°$ in water (*c*, 1·6). (Found: C, 40·8; H, 6·9; N, 11·5; OMe, 26·0. $C_8H_{16}O_6N_2$ requires C, 40·7; H, 6·8; N, 11·9; OMe, 26·3 %.)

The known compound (name in roman) had m.p. 178–179°, unchanged by admixture with an authentic sample kindly supplied by Dr Z. (Found: C, 48·6; H, 6·1; OMe, 50·1. Calc. for $C_{10}H_{16}O_7$: C, 48·4; H, 6·4; OMe, 50·0 %.) *Alternatively*: The known compound had m.p. 178–179°. The mixed m.p. with an authentic sample (m.p. 179–181°) prepared according to X & Y (1932) was 178–180°. (Found: C, 49·4; H, 3·8; N, 3·9; loss at 100°, 5·1. Calc. for $C_{28}H_{22}N_2I_2$, $2H_2O$: C, 49·7; H, 3·85; N, 4·15; H_2O, 5·3 %.)

Distillation of the product gave a middle fraction (0·3 g.), b.p. 120°/15 mm., n_D^{16} 1·4767; light absorption max., 2260 and 2650 Å (ϵ 2200 and 2500 respectively) (Found: C, 65·0; H, 8·5. Calc. for $C_{10}H_{16}O_3$: C, 65·2; H, 8·75 %), which was probably a mixture of *A* and *B*.

Notes

1. *Melting points.* It is desirable to state whether m.p.'s are corrected or uncorrected for the emergent stem of the thermometer. This can often be done conveniently at the beginning of the experimental section.

2. *Specific optical rotations.* It is desirable to give an estimate of the error involved.

3. *Percentages in elementary analyses.* These should generally be given to one place of decimals only, since the second place is rarely significant.

4. Elements are given in the order C, H, O, N, Cl, Br, I, F, S, P and then the remainder in alphabetical order of symbols.

METABOLIC QUOTIENTS

1. (*a*) The symbol Q_x may be used to represent metabolic quotients only in the units μl. X/mg. dry wt. of biological material/hr. (*b*) If the metabolite X is a solid or liquid it is for the purpose of this convention considered as a gas at s.t.p., 1 μmole of X being equivalent to 22·4 μl.

2. Metabolic quotients in any other units, e.g. μmole X/mg. dry wt./hr.; μl. X/mg. N/sec., may be represented by the symbol q_x.

3. The units for Q or q must be clearly defined at the first mention in each paper.

4. Production and removal of metabolites are indicated by quotients with + and − signs respectively. The signs may be omitted if no confusion can thus arise.

5. Aerobic or anaerobic conditions may be indicated by the superscripts O_2 and N_2 respectively, thus: $Q_{CO_2}^{O_2}$, $Q_{CO_2}^{N_2}$, $q_{CO_2}^{O_2}$, $q_{CO_2}^{N_2}$. When measurements are made in another gas, this may be indicated by the appropriate superscript, e.g. $Q_{CO_2}^{H_2}$.

6. The substrate may, if necessary, be indicated in parentheses following the Q symbol, thus $Q_{CO_2}^{N_2}$ (pyruvate).

7. It is sometimes preferable not to use the special symbols Q_x or q_x, but to state units (e.g.) μmole/g. wet wt./hr.) each time a metabolic quotient is mentioned.

CHEMICAL NOMENCLATURE

Order of prefixes. An alphabetical order will be followed, cf. *J. chem. Soc.* 1950, p. 3699. Syllables indicating multiple substituents, di-, tri-, do not count in deciding the order. Thus, 2-ethyl-1-methylnaphthalene; 2-ethyl-1:4-dimethylnaphthalene.

Use of hyphens and italics. All names are run together except for those of ethers, glycosides, ketones, acids, salts and esters, which are printed as separate words, e.g. aminonaphtholsulphonamide, but ethyl methyl ketone, ethyl acetate, cetyltrimethylammonium bromide, methyl glucoside.

No hyphens are used except to separate numbers or Greek letters or some italic prefixes from words—thus, 2-ethylhexanol, *n*-butanol, $\beta\beta$-dimethyl-D-cysteine, 5α-pregnan-3β-ol.

Italics are used for certain prefixes so that these shall not be counted as part of the name for indexing—e.g. *n-*, *iso*, *sec-*, *tert.-*, *cis-*, *trans-*, *allo*, *dextro*, *laevo*, *meso*. They are also used for *O-*, *N-*, etc., when they are used to indicate the atom carrying a substituent, e.g. in *N'*-2-pyridylsulphanilamide.

Isotopically labelled organic compounds. The isotopic symbol and, where necessary, the positional number of the atom labelled are given in square brackets before the name of the compound. Thus, for example, [α-^{14}C]alanine; $CH_3.{}^{14}CH(NH_2).CO_2H$; [*carboxy*-^{13}C]acetic acid; $CH_3.{}^{13}CO_2H$.

A draft of editorial proposals of the Chemical Society and the Biochemical Society is used as the authority for nomenclature; in difficult cases the *Biochemical Journal* editorial office will be prepared to assign names.

Amino acids. See *Biochem. J.* 1948, **42**, 1; 1952, **52**, 1.

Carbohydrates. Many changes in nomenclature have been made as a result of discussions between British and U.S. chemists. See *J. chem. Soc.* 1952, p. 5108.

Steroids. The Ciba Foundation rules, *J. chem. Soc.* 1951, p. 3526 will be used.

Vitamins. See *Biochem. J.* 1952, **52**, 1. The terms 'Vitamin A', 'B', etc., may still be used where it is desired to emphasize the biological activity and not the chemical nature of a material.

Organophosphorus compounds. See *J. chem. Soc.* 1952, p. 5122.

Formulae. These should, if possible, be written in one line since this saves space and expense in printing. Dots are used to represent the links in the main chain; side chains are in parentheses, and condensed main chains are in square brackets, thus, e.g.:

$$CH_2{:}CH.CH(OH).CH_3,$$
$$NH_2.[CH_2]_3.CH(NH_2).CO_2H.$$

Structural formulae are best reproduced from blocks. If authors cannot produce an indian ink drawing from which a block can be made directly, they should make a careful pencil drawing which can be copied by a draughtsman. A charge for redrawing by the Press may be made to the author. Hexagons and preferably also pentagons should be regular. Hetero atoms should be shown in the ring. Aromatic rings must show double bonds. Lettering should be in pencil.

The Application of Countercurrent Methods to the Fractionation of Lipid Material from Human Placenta

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The method of countercurrent fractionation has been applied to the separation of a wide variety of substances. Among animal lipids, Lovern (1952) has studied ox-brain phospholipids, Lovern & Olley (1952) fractionated haddock-muscle lipids, while the lipids of human brain have been investigated by Cole, Lathe & Ruthven (1953). The latter study has now been extended to the lipid components of human placenta.

The composition of placental lipids has been estimated previously by Pratt, Kaucher, Richards, Williams & Macy (1946) using ordinary chemical procedures. This work involves the assumption that the types of lipid found in placenta do not differ widely from those of brain, for which most of the analytical methods have been perfected. In the work now reported it has been found that in using the countercurrent method, lipid fractions obtained from placenta differ from those of brain. In particular, it seems impossible to account for the nitrogen and phosphorus contents of the placenta lipids on the basis of the types of phospholipid predominating in brain.

METHODS

The methods of countercurrent distribution of lipid, together with analytical and paper-chromatographic procedures, have already been published (Cole *et al.* 1953), and the apparatus used was that of Lathe & Ruthven (1951). Unless otherwise stated, distributions were made through 49 transfers. Figures of the distributions are drawn with the tubes containing the polar material to the left.

Extraction. Placental lipids were extracted from whole, full-term, organs by the method of Murray, Lathe, Clark, Ruthven & Levine (1950). The membranes and cord were detached, and the placentae treated within 2–10 hr. of delivery.

Solvent systems. The solvent systems are given in Table 1. Systems used previously (Cole *et al.* 1953) are referred to by the same letters.

Partition coefficient of neutral fat. About 55 mg. of neutral fat (prepared from beef suet) was equilibrated between 10 ml. of each of the phases of system *A*. The two layers were separated, and equal volumes taken to dryness *in vacuo*. The partition coefficient was 0·092.

EXPERIMENTAL

Distribution of crude placental lipid

Two schemes of fractionation were used. The first (Fig. 1) was similar to that used for brain lipid. After each distribution the solvent was evaporated from every tube, and the residue was dried and weighed. As this probably involved some damage to the lipid through oxidation or decomposition, scheme 2 (Fig. 2) was devised. Here two samples of lipid were distributed in a solvent system suitable for polar materials. One of the sets of fractions was taken to dryness and weighed, and on the basis of these weights the second set was divided into three groups of tubes, each of which was combined, and redistributed in an appropriate new solvent system. In this way repeated drying of individual fractions was avoided.

Scheme 1. Fig. 1*a* shows the countercurrent distribution, through 49 transfers, of 1·25 g. of crude placental lipid in solvent system *A*. Three main groups of fractions were evident: tubes 0–16, 17–38, and 39–49. These were of polar, intermediate, and non-polar character, and weighed 20, 63 and 17% of the whole, respectively. In order to obtain larger amounts of these materials for redistribution, 8·7 g. of crude lipid were distributed, in flasks, through eight transfers in system *A*, using 100 ml. of each phase. After

Table 1. *Solvent systems for countercurrent distributions*

(Figures in the body of the table are volumes of individual components required to prepare system; LP, light petroleum, b.p. 40–60°.)

System	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*H*
Volume ratio of upper to lower phase	1/1	1/4	1/1	1/1	1/2	1/8	2/1	4/1
Components	CCl_4 62 CH_3OH 35 Water 3·15	As system *A*	LP 31 CCl_4 31 CH_3OH 35 Water 3	CCl_4 25 $CHCl_3$ 15 CH_2Cl_2 10 CH_3OH 40 Water 10	As system *D*	As system *D*	As system *A*	As system *A*

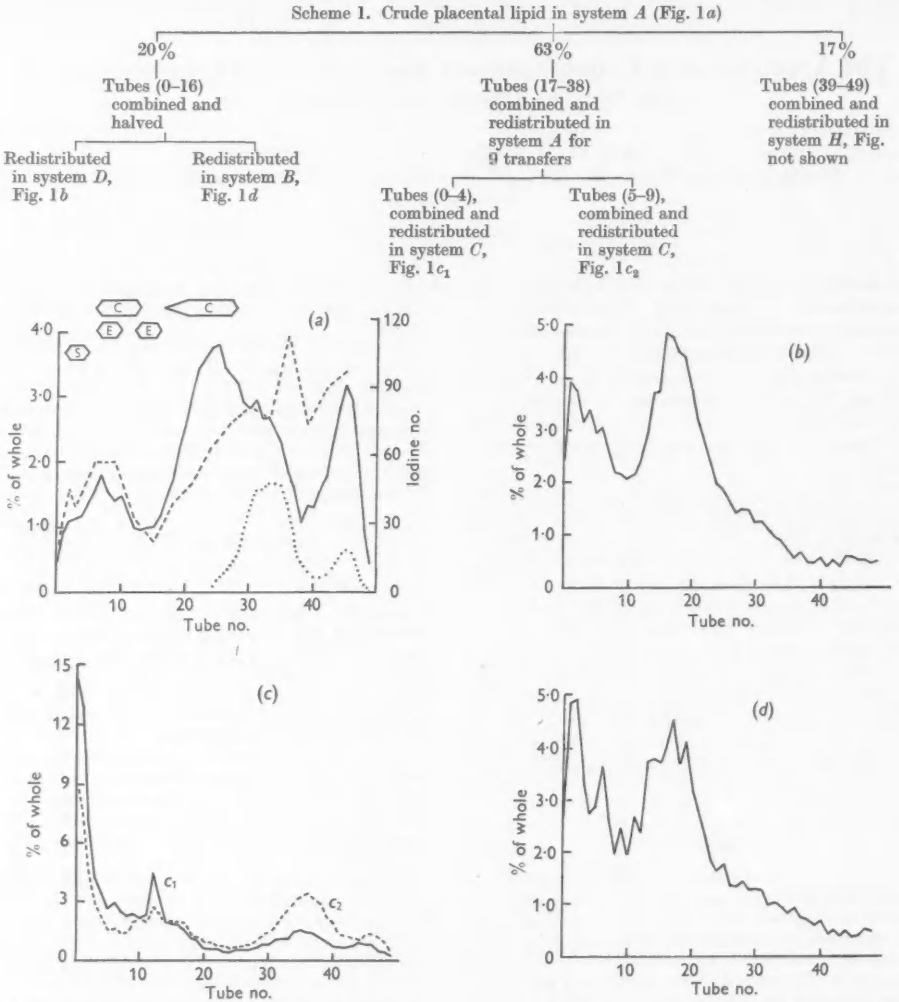


Fig. 1. (Scheme 1.) Distributions of placental lipid. (*a*) Distribution of crude placental lipid in system *A* by weight (——), showing the position of cholesterol (. . . .) and the iodine number of the non-cholesterol material (- - -). (Bases are shown as follows: ethanolamine (E), choline (C), serine (S).) (*b*) Distribution of half of the combined polar fractions of crude placental lipid (tubes 0–16 of *a*) in system *D*. (*c*) Distributions (c_1) (——) and (c_2) (- - -) in system *C* of tubes (0–4) and (5–9) respectively of a 9-transfer distribution in system *A* of the intermediate fractions of tubes 17–38 of *a*. (*d*) Distribution of half of the combined polar fractions of crude placental lipid (tubes 0–16 of (*a*)) in system *B*.

removing the solvent *in vacuo* and reconstituting each tube to 20 ml. of each phase, the distribution was continued in the countercurrent apparatus for an additional 23 transfers. The fractions were then divided into three groups, namely, tubes 0–10, 11–24 and 25–31, based on the divisions of the 49-tube distribution. As the fractions were combined a graph of the 31-transfer distribution is not shown. In Fig. 1*a* the division of this distribution is referred directly to the 49-tube distribution, which is more suitable for comparisons.

Before distributing the polar group of fractions (tubes 0–16 of Fig. 1*a*) in new solvents they were distributed again in solvent *A* through 24 transfers. The result is not shown, as little further separation took place, 87·5% of the material

remaining in tubes 0–8. This was combined (the other 12·5% being added to the lipid of intermediate polarity in tubes 17–38 of Fig. 1*a*), and halved. One half was distributed in solvent system *D* (Fig. 1*b*) and the other in solvent system *B* (Fig. 1*d*). These two solvent systems represent alternative methods of moving the polar fractions toward the centre of the distribution. In each case the lipid was split into two groups (tubes 0–10 and 11–25). As they were not yet centrally placed later studies (Scheme 2) utilized the combined effect of changing the solvents and altering the ratio of phases.

The intermediate fractions (tubes 17–38 of Fig. 1*a*), after being combined, were given a preliminary distribution in the same system *A* for 9 transfers. Tubes 0–4 and 5–9 were then combined, and the solvent removed from each. The two materials were then distributed separately in system *C* (Fig. 1c_1 and 1c_2). This resulted in greater differentiation, both distributions showing the following groups of fractions: tubes 0–5, 6–26, 27–42 and 43–49. As would be expected from the origin of the materials, there was a preponderance of non-polar lipid in distribution c_2, and of the polar lipid in c_1.

The non-polar fractions (tubes 39–49 of Fig. 1*a*) from the distribution in system *A* were redistributed in system *H*. The graph, which is not included, showed that the components were not separated but remained as one group in tubes 30 to 45.

system (Fig. 1*c*), tubes 0–24 of this distribution were taken to dryness and weighed individually, while tubes 25–49 were combined. Most of the weighed material was found in tubes 10–24 with a peak at 16 (Fig. 2*d*). The combined, non-polar part was redistributed in system *G*, giving two groups of fractions in tubes 12–30 and 31–49 (Fig. 2*e*).

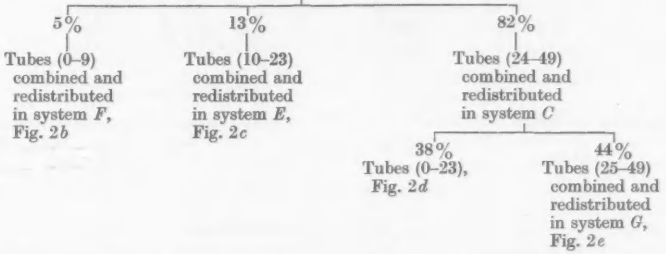
Characterization of countercurrent fractions from placental lipid

The nature of the various fractions produced by countercurrent distribution has been investigated by chemical analyses. In addition, the contents of each tube were hydrolysed with acid, and paper chromatography applied to the identification of bases and phosphates.

Cholesterol. The crude placental lipid contained approximately 16% of cholesterol, according to the Liebermann-Burchard reaction. Estimation of cholesterol in the individual tubes of a distribution in solvent *A* (Fig. 1*a*) showed that cholesterol occurred in tubes 24–49, with peaks at tubes 34 and 45, which contained 70 and 20% cholesterol, respectively. The partition coefficient of cholesterol in system *A* (Cole *et al.* 1953) was calculated to give a peak at tube 35. The less polar of the two peaks, at tube 45, may be attributed to cholesterol esterified with fatty acids.

Cholesterol was estimated in the two distributions in solvent *C* (Fig. 1*c*). The cholesterol curve, which is not

Scheme 2. Crude placental lipid in system *B* (Fig. 2*a*)


5%
13%
82%
Tubes (0–9) combined and redistributed in system F, Fig. 2b
Tubes (10–23) combined and redistributed in system E, Fig. 2c
Tubes (24–49) combined and redistributed in system C
38%
44%
Tubes (0–23), Fig. 2d
Tubes (25–49) combined and redistributed in system G, Fig. 2e

Scheme 2. About 3 g. of crude placental lipid were distributed in solvent system *B*, and taken to dryness. On the basis of the weight curve (Fig. 2*a*) division into the following groups seemed appropriate:

Character of lipid	Tube nos.	% of whole
Very polar	0– 9	6
Polar	10–23	12
Intermediate and non-polar	24–49	82

Accordingly, a larger scale distribution of 6 g. of crude lipid in solvent system *B*, using 100 ml. of solvent per tube, was run through 49 transfers, after which the tubes were combined according to the above table, and then each of the three solutions was reduced in volume for redistribution in other systems.

The redistribution of the very polar fractions (tubes 0–9 of Fig. 2*a*) in system *F* spread the bulk of the material widely (Fig. 2*b*). The slightly less polar lipid (tubes 10–23 of Fig. 2*a*) when distributed in system *E* was found mainly in tubes 18–32, with a peak at tube 25 (Fig. 2*c*). The remaining fraction of Fig. 2*a* (tubes 24–49) was distributed in system *C* (Fig. 2*d*). In view of the previous experience with this shown, had a peak at tube 35, and traces were present in tubes 6–30. There was no peak at tube 45, nor was this to be expected since the non-polar fractions had been separated before these distributions were made.

In system *G*, scheme 2, the position of cholesterol is shown in Fig. 2*e*. Peaks were found at tubes 27 and 39, containing 70 and 24% of cholesterol, respectively. This was in agreement with the position of the cholesterol peaks in system *G* when calculated from the maxima found in system *A*. The shape of the less polar of these peaks indicated that more than one compound of cholesterol was present.

Neutral fat. The partition coefficient of a specimen of neutral fat (not of human placental origin) was found to be 0·09 in system *A*. From this it was calculated that neutral fat would form a peak at tube 45 in a distribution of 49 transfers. Such a peak, representing 17% of the original lipid, was present in Fig. 1*a*. However, it contained some cholesterol and P as well. In system *G* the theoretical peak of neutral fat would be at tube 42, which coincides with the non-polar peak of Fig. 2*e*.

Degree of unsaturation. Since oxidation could not be prevented completely, the values found for unsaturation may not be of great significance, but they should reveal the

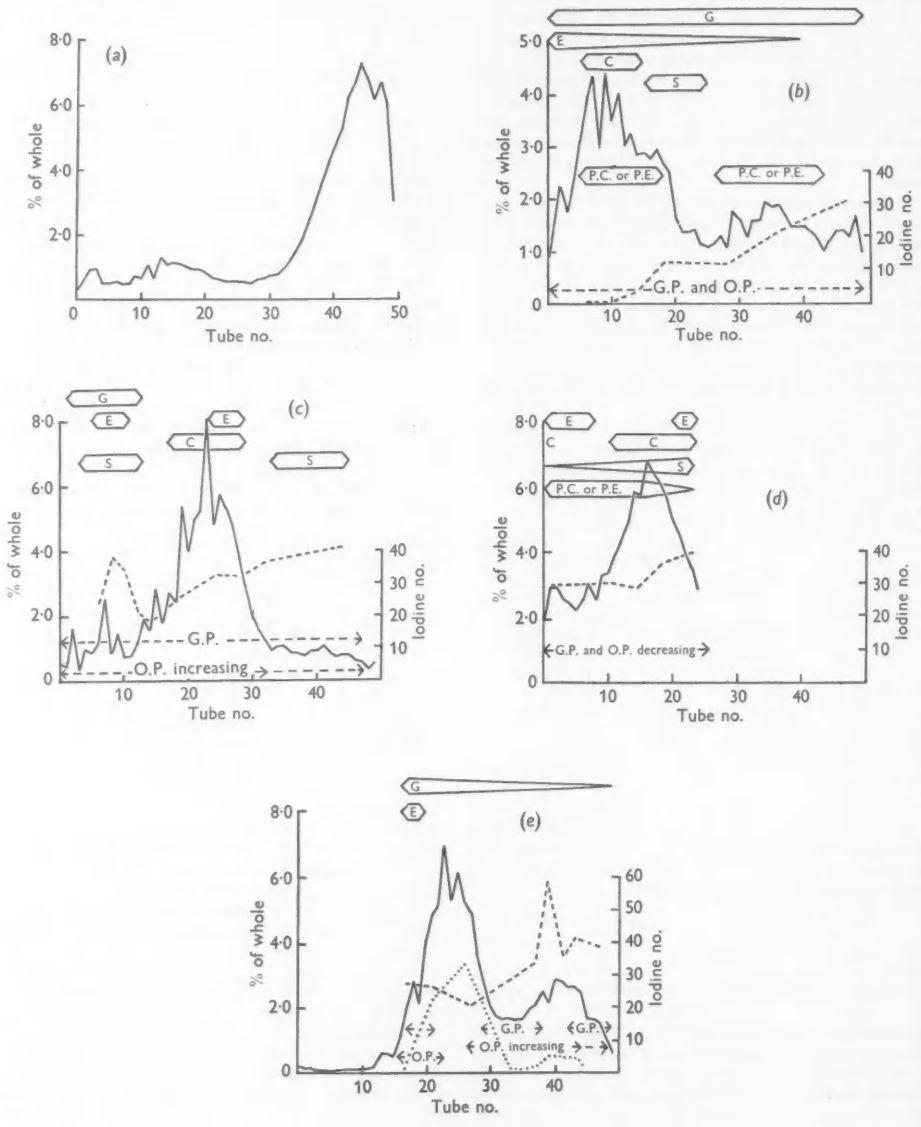


Fig. 2. (Scheme 2.) Distributions of placental lipid by weight (——) showing cholesterol (. . . .) and the iodine number of the non-cholesterol material (- - -), glutamic acid (G), ethanolamine (E), choline (C), serine (S), glycerophosphate (G.P.), orthophosphate (O.P.), phosphoryl choline (P.C.) and phosphoryl ethanolamine (P.E.). (*a*) Distribution of crude placental lipid in system *B*. (*b*) Distribution of the most polar fractions of crude placental lipid (tubes 0–9 of *a*) in system *F*. (*c*) Distribution of the polar fractions of crude placental lipid (tubes 10–23 of *a*) in system *E*. (*d*) Distribution of fractions of intermediate polarity and non-polar fractions of crude placental lipid (tubes 24–49 of *a*) in system *C*. (*e*) Distribution of the non-polar fractions of crude placental lipid (tubes 25–49 of *d*) in system *G*.

relative degree of unsaturation. In all calculations allowance was made for cholesterol, and the values given refer to the iodine number of the non-cholesterol material.

Inspection of the iodine numbers showed that the very polar material (Figs. 1*a*, 2*b*) was relatively unsaturated. Among the materials of intermediate polarity in Fig. 1*a* a steep gradient appeared between tubes 15 and 36. However, in the distributions of comparable materials in scheme 2 (Fig. 2*d*, *e*) this was absent.

Nitrogen and phosphorus analyses. The distribution of lipid in system *A* (Fig. 1*a*) was examined for P, while N analyses were made on a second similar distribution. For the purpose of comparing amounts of N and P with weights of non-cholesterol lipid it has previously been found convenient to chart P in terms of the amount of phosphatide to which it was equivalent, using the factor $P \times 100/3{\cdot}85$. Similarly, a figure of $N \times 100/1{\cdot}8$, based on monoaminophosphatides, has been used for charting N. Fig. 3 is a composite graph of these data, together with a curve of the mean of the non-cholesterol material in the two distributions.

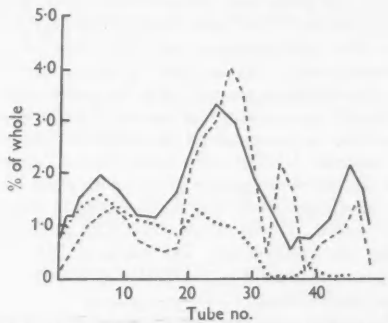


Fig. 3. Nitrogen and phosphorus content of the non-cholesterol material (——) of crude placental lipid distributed in solvent *A* (compare Fig. 1*a*). Nitrogen (. . . .) and phosphorus (- - -) are expressed as equivalent amounts of monoaminophosphatides ($N \times 100/1{\cdot}8$ and $P \times 100/3{\cdot}85$).

The most striking feature of the results was the disparity between the phosphatide curve based on the P content, and that calculated from N. The more polar components (tubes 0–15 of Fig. 3) had a N content comparable with monoaminophosphatides, while the P content was too low in this region. In the area of intermediate polarity (tubes 15–30 of Fig. 3) N declined and the P content became much greater than could be attributed to phosphatides of the usual type. Moreover, considerable amounts of P occurred in the non-polar regions, with peaks in tubes 35 and 46, which contained no N.

Identification of bases. The contents of the individual tubes from the distribution of placental lipid in system *A* (Fig. 1*a*), as well as the distributions from scheme 2, were hydrolysed with acid, desalted, and the bases were identified by paper chromatography using two solvent systems. The findings are shown in Figs. 1*a* and 2*b–e*.

The small amounts of material in the most polar tubes (0–4) of the distribution in system *A* (Fig. 1*a*) made chromatographic identification difficult. The only amino acid found here was serine. However, in comparable fractions from scheme 2 (Fig. 2*b*) glutamic acid was found. Glutamic acid also appeared in the polar tubes of Fig. 2*c*, and also in the less polar materials (Fig. 2*e*).

The three phosphatide bases, choline, serine and ethanolamine, were observed widely spread throughout the polar tubes and those of intermediate polarity (Fig. 2*b–d*), giving the impression of many different fractions. The non-polar material (Fig. 2*e*) was free of bases except for a little ethanolamine about tube 20.

Chromatograms stained for phosphate. The distributions of scheme 2 were examined for P compounds by paper chromatography, after acid hydrolysis of the fractions. Inorganic P and glycerophosphate were found throughout the fractions. The amount of inorganic P decreased towards the non-polar end of all the distributions except that of the most non-polar material in system *G* (Fig. 2*e*). Here there was a relative increase of orthophosphate and glycerophosphate toward the non-polar end of the distribution, but very little was present in the region of the free cholesterol peak (tubes 20–30). Phosphatidylcholine or phosphatidylethanolamine (which were not distinguished easily in the systems used) also appeared in tubes 3–17 and 26–43 of the distribution in system *F* (Fig. 2*b*), in tube 29 of system *E* (Fig. 2*c*) and tubes 0–24 of system *C* (Fig. 2*d*).

DISCUSSION

Human placenta is an organ of rather diverse structures, which includes many blood vessels and much loose connective tissue, in addition to the trophoblast. It is not surprising then that placental lipids appear to be at least as complex as those of brain (Cole *et al.* 1953).

The lipid was prepared from placentae contaminated with maternal and foetal blood and thus contained a certain amount of fat-soluble material from these sources. However, a comparison of the lipid composition of washed placenta, as determined by Pratt *et al.* (1946), with the lipid composition of blood, as estimated by Boyd (1933), shows that the main effect of contamination with blood is to increase the placental content of free and ester cholesterol.

In scheme 2 manipulation, particularly warming and drying, was reduced to a minimum in order to avoid oxidation and decomposition of the lipid. Apart from the known lability of lipid materials, the need for these precautions was suggested by a number of observations, including the presence of a large polar peak (approximately 35% of the distributions) in Fig. 1*c* of scheme 1. This was difficult to explain other than by decomposition. The absence of this peak in the corresponding distribution (Fig. 2*d*) of scheme 2 strengthens this impression. Apart from this difference between the two schemes, the general pattern of the lipid distributions is very similar.

The composition of the lipids of placenta and of brain can be compared, since the initial distributions were made under the same conditions (Fig. 1*a*, this

paper, and Fig. 4*a*, Cole *et al.* 1953). A similarity in composition is shown by peaks in both distributions at about tube 10, and in the central region. However, there were three main differences. First, placenta contained a large fraction (equivalent to 17 % of the crude lipid) in the non-polar region (tubes 39–49, Fig. 1*a*) in the position of neutral fat. While neutral fat is almost absent from brain, human placental lipid contains 30 %, according to Pratt *et al.* (1946). The fact that the non-polar peak of Fig. 1*a* also contained cholesterol and phosphorus accentuated the quantitative difference between our findings and those of Pratt. A possible explanation of this discrepancy may lie in the fact that Pratt estimated neutral fats from the amount of acetone-soluble glycerol, since recently Lovern & Olley (1952) demonstrated that in at least one tissue, haddock muscle, an important proportion of the phosphatide is acetone-soluble.

A second difference between placenta and brain was found in the centre of the distributions (tubes 15–35). Here the greater prominence of the placental curve on the polar side may be attributed to its greater content of lecithin, while the smaller amount of material in the region of tube 29 of the placental curve may be due to the larger amount of cerebroside in brain. On the non-polar side of the central peak cholesterol was present in equal amounts (16 %) in brain and placenta. The fact that this was higher than reported for placenta by Pratt *et al.* (1946), was probably due to contamination with blood in our preparations. As regards sphingomyelin, which in brain is thought to have a peak at tube 21, we have no data for placenta.

Thirdly, in placenta there was no peak corresponding to the prominent polar fractions found in the first five tubes of the brain distribution, and characterized by the presence of much reducing material and many amino acids.

The distribution of bases in placenta and brain can be compared (Fig. 1*a*, this paper, and Fig. 5, Cole *et al.* 1953). In addition to the absence in the placental distribution of the polar, amino-acid-containing fraction (tubes 0–4), it was noted that while choline was present only in the central fractions of brain, in placenta it occurred not only there but also in the more polar tubes (5–11). This latter position probably corresponds with the main concentration of choline which Lovern (1952) found in distributions of ox-brain phosphatide. Serine was detected in placenta over a much smaller area of the distribution in system *A* than it was in brain, although the more detailed study of bases made in scheme 2 showed that here it was much more widely distributed. The distributions of bases, scheme 2, indicated the presence of many compounds, and suggested that the placental lipids were very complex. There were three or four areas of high ethanolamine concentration, three of choline and two or three of serine. Glutamic acid occurred in all the distributions except that of the central fractions (Fig. 2). This amino acid was noted in the most polar brain fractions by Cole *et al.* (1953), and it has also been found in kephalin prepared from brain (Hecht & Mink, 1952). Wynn & Williams (1950), who found glutamic acid, together with other amino acids, in light petroleum extracts of plasma, considered that it occurred as part of a peptide combined with plasma lipid. The widespread occurrence of glutamic acid in the placental lipid distributions reported here suggests that there is some general relationship between this amino acid and the placental lipids, rather than the existence of a definite glutamic acid-containing lipid.

Some rather unexpected features were presented by the nitrogen and phosphorus analyses of placenta. In brain, these two elements were present only in the polar and intermediate tubes, and at concentrations which were in reasonable agreement with the predominance of phosphatides. In placenta, however, as shown in Fig. 3, the concentration of phosphorus was too low in the polar material, and much too high in the intermediate and non-polar lipid, to be explained on this basis. The nitrogen content, on the other hand, was comparable with that of phosphatides in the more polar half of the distribution, but was much lower in the non-polar region. It was also noticed that in the areas where the phosphorus was high, a qualitative Feulgen reaction for aldehyde appeared to be particularly strong.

The identity of these high-phosphorus compounds has not been established. Acetal phosphatides (Thannhauser, Boncoddo & Schmidt, 1951) may be present, as the reaction for aldehyde suggests, but since the areas with high phosphorus content are low in nitrogen, plasmalogens can account for only a small proportion of the phosphorus. On the other hand, compounds like cardiolipin (Pangborn, 1947), phosphatidylinositol (Folch, 1942), and phosphatidic acids (Chibnall & Channon, 1929), although devoid of nitrogen, have approximately the same phosphorus content as the monoaminophosphatides. Diphosphoinositide (Folch, 1949), contains more phosphorus (7·3 %) and negligible nitrogen. However, from its properties, a much more polar position in the distribution would be expected. It seems more probable that phosphate-containing lipids of a new type occur in the placenta, although the possibility that they have been produced by hydrolysis of phospholipids between delivery and extraction of the lipids, might be considered.

Recent studies have tended to emphasize the dynamic role played by the placenta in the nutrition of the foetus. This has been demonstrated in relation to both fat (Popják & Beeckmans, 1950) and carbo-

hydrate metabolism (Huggett, Warren & Winterton, 1949) as well as by the high overall rate of oxygen consumption of the tissue (Wang & Hellman, 1943). The pattern of placental lipids, and in particular the small proportion of neutral fats, and the abundance of compounds which are high in phosphate, is in agreement with the conception of the placenta as an actively metabolizing tissue. Together with the complexity of the phospholipids, as indicated by the distribution of bases, these are the most striking features of the lipid components of placenta.

SUMMARY

1. The lipids of mature human placentae have been fractionated by countercurrent distribution in a solvent system of carbon tetrachloride, methanol and water, which was further modified for use with polar and non-polar lipids.

2. The placental lipids have been compared with those of brain, distributed under the same conditions.

3. Ethanolamine, choline and serine each occur in several fractions of placental lipid.

4. Glutamic acid is distributed so widely as to indicate some general relation to the placental lipids.

5. The nitrogen and phosphorus contents are not consistent with the predominance of the usual phospholipids, but indicate that compounds of high phosphorus and low nitrogen content are present.

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The Oxidative Pathway of Carbohydrate Metabolism in *Escherichia coli*

1. THE ISOLATION AND PROPERTIES OF GLUCOSE 6-PHOSPHATE DEHYDROGENASE AND 6-PHOSPHOGLUCONATE DEHYDROGENASE

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It has been shown that some bacterial viruses change the metabolism of the host cell in such a way that virus deoxyribosenucleic acid is synthesized instead of host deoxyribosenucleic acid and ribosenucleic acid. The hypothesis has been advanced that the routes of carbohydrate metabolism leading to the production of pentose and deoxypentose were different and that the relative amounts of glucose used by the two pathways were changed during elaboration of virus by the host (Cohen, 1947, 1951*a*). The glycolytic, or Embden-Meyerhof, breakdown of one molecule of glucose 6-phosphate gives rise to two molecules of triosephosphate. It has been shown by Racker (1951) that condensation of glyceraldehyde phosphate with acetaldehyde by enzymes from *Escherichia coli* and other cells yields deoxyribose 5-phosphate which may be used in the synthesis of deoxyribosenucleic acid.

By the oxidative, or Warburg-Lipmann-Dickens pathway (Warburg, Christian & Griese, 1935; Lipmann, 1936; Dickens, 1938), oxidation of glucose 6-phosphate yields 6-phosphogluconate. It

has been shown in this laboratory that ribose 5-phosphate is a product of the degradation of 6-phosphogluconate by enzymes isolated from yeast (Scott & Cohen, 1949; Cohen & Scott, 1950) or from *Escherichia coli* (Scott & Cohen, 1951; Cohen, Scott & Lanning, 1951). Evidence has been adduced by Horecker & Smyrniotis (1950) that this ribose 5-phosphate is derived from the isomerization of ribulose 5-phosphate, which is the more immediate product of the oxidative decarboxylation of phosphogluconate. It has also been shown (Horecker & Smyrniotis, 1952) that ribulose 5-phosphate can be broken down by enzymes to produce triose phosphate and an as yet unidentified two-carbon fragment. The reversibility of this reaction has not yet been demonstrated.

Studies in this laboratory on the utilization of [1-^{14}C]glucose by *Esch. coli* gave values for recovery of the labelled carbon in carbon dioxide which indicated a use of approximately 37 % of the glucose by the oxidative pathway during growth, and a shift of some of this glucose to the glycolytic pathway on infection with bacteriophage (Cohen, 1951*b*). Thus it seemed that the use of the phosphogluconate pathway was reduced as ribosenucleic acid synthesis was inhibited in the presence of the virus.

A study was undertaken to determine whether the dehydrogenases for glucose 6-phosphate and for 6-phosphogluconate were present in the bacterial cells in amounts sufficient to account for the apparent use of glucose by the oxidative pathway, as indicated by the isotopic data. In order to determine the amounts it was necessary to be able to assay these enzymes under optimal conditions. The determination of these conditions is the subject of this paper.

METHODS AND MATERIALS

The activities of the enzymes were determined in the Beckman spectrophotometer with a thermostatically controlled cell chamber. The increase in density at 340 mμ. caused by the reduction of triphosphopyridine nucleotide (TPN) was measured at 0·5 or 1 min. intervals after the reaction was started by addition of the last component of the system. The reaction was carried out in 1 ml. vol. in a cuvette with a 1 cm. light path. Results are expressed as the increase in density (ΔE)/ml. of extract/min., or increase in density/mg. of protein/min., or as μmole TPN reduced/mg. protein/min. Unless it is otherwise stated, all estimations were carried out at 36°.

Protein was determined by a modification of the Nessler procedure (Miller & Miller, 1948), or by the precipitation of the protein in 2·5 % perchloric acid. With the latter method, turbidity was read at 420 mμ. in a Klett photometer. The relation of turbidity to protein was linear and reproducible for 0·3–1·5 mg. protein in a total vol. of 5 ml. In addition, the ratios of the u.v. absorptions at 260 and 290 mμ. were useful to indicate what proportion of nucleic acid was present in the protein solutions.

TPN was prepared by the method of LePage & Mueller (1949). Fractions eluted from the charcoal column were 37–55 % pure by enzymic assay and contained no diphosphopyridine nucleotide.

Glucose 6-phosphate was prepared by the method of Seegmiller & Horecker (1951). The synthetic Ba salt was 80 % pure by enzymic analysis. 6-Phosphogluconate, prepared by oxidation of either the natural or the synthetic glucose 6-phosphate, contained 81 % phosphogluconate and was free of glucose 6-phosphate.

The buffers used were glycylglycine or tris(hydroxymethyl)aminomethane (Tris) (Gomori, 1946).

The enzymes were prepared from *Esch. coli*, strain B, grown at 36° in a mineral medium (Cohen, 1949) of pH 7·8 to which glucose or gluconate was added as the sole source of carbon. For maximal aeration, the bacteria were grown in roller flasks revolving at 300–400 rev./min. Growth was followed by measurement of the turbidity of the culture with the 420 mμ. filter in the Klett photometer. The number of cells was determined either by plating or from a standard curve for turbidity correlated with plate count.

Cells were harvested at the desired stage of growth, or immediately after growth had stopped because of exhaustion of the carbon source. The bacterial suspension was cooled in an ice bath, and all further procedures were carried out at 4°. The bacteria were centrifuged, washed with 0·85 % saline or saline containing 0·1 % glucose, and centrifuged again at 1800 *g* for 15 min. The wet pellet of bacteria was weighed and ground with 2·5 times its weight of alumina (Alcoa A 301) for 2 min. and then extracted with the desired volume of water or buffer (McIlwain, 1948). Phosphate buffer at pH 7·4 extracted more glucose 6-phosphate dehydrogenase and less phosphogluconate dehydrogenase. Extraction with glycylglycine or with water yielded less of the former and more of the latter enzyme. Of the solutions tried, Tris buffer was the best for extracting both enzymes (see Table 1). The extract was obtained by centrifugation in the cold at 10000 *g* for 30 min. Centrifugation for 2 hr. at 10000 *g* or for 1 hr. at 100000 *g* did not sediment any of the activity in the water extracts. The alumina pellet was re-extracted once or twice. The activities of these extracts in reducing TPN in the presence of glucose 6-phosphate (G 6-P) and phosphogluconate (PG) were assayed immediately. Typical preparations are shown in Table 1.

Separation and partial purification of the enzyme

The extracts (Table 2, *O*) containing about 5–10 mg. protein/ml. were treated with 0·1 vol. of 0·25 M-$MnCl_2$ added slowly with constant agitation. After standing 30 min., the precipitated nucleic acid was removed by centrifugation. Little or no enzyme activity was lost in this step.

Saturated $(NH_4)_2SO_4$ at pH 7·6 was added to the supernatant fluid (*S*) to 45–50 % saturation. The slight precipitate (*A*) was removed by centrifugation and discarded.

Glucose 6-phosphate dehydrogenase. To the supernatant fluid, $(NH_4)_2SO_4$ was added to 60 % saturation. The precipitate (*B*) was dissolved in water and reprecipitated at 60 % saturation of $(NH_4)_2SO_4$. This precipitate (*C*) contained 35–80 % of the total glucose 6-phosphate dehydrogenase activity of the extract and 2 % or less of phosphogluconate dehydrogenase. This step accomplished a 3- to 7-fold increase in activity/mg. of protein. Dialysis of the enzyme against running cold distilled water caused precipitation and loss of 60–80 % of the activity. However, the enzyme could be dissolved in buffer or albumin solution and

Table 1. *Extraction of protein and enzymes*

(For details, see text.)

Wet weight of bacteria (g.)	Extractant	Vol. (ml./g.)	Extract	Protein (mg./ml.)	Glucose 6-phosphate dehydrogenase (ΔE/mg./min.)	Phosphogluconate dehydrogenase (ΔE/mg./min.)	% of total extracted: Protein	% of total extracted: Glucose 6-phosphate dehydrogenase	% of total extracted: Phosphogluconate dehydrogenase	Ratio: Glucose 6-phosphate dehydrogenase / Phosphogluconate dehydrogenase
Exp. 1										
1·00	Water	10	First	11·4	1·27	0·79	—	—	—	0·63
1·00	Glycylglycine, 0·1 M, pH 7·4	10	First	11·2	1·72	0·66	—	—	—	0·38
1·00	Tris, 0·1 M, pH 7·4	10	First	10·7	1·85	0·75	—	—	—	0·40
1·00	Tris, 0·1 M, pH 8·0	10	First	10·0	1·78	0·86	—	—	—	0·45
1·00	Tris, 0·1 M, pH 8·7	10	First	10·8	1·57	0·85	—	—	—	0·54
Exp. 2										
2·28	Water	2	First	48·5	1·29	0·91	59	81·5	65	0·71
		8	Second	6·53	0·47	0·71	35	17·5	30	1·5
		10	Third	0·95	0·10	0·63	6	0·6	4·7	6·3
Exp. 3										
1·64	Tris, 0·1 M, pH 8·0	5	First	27·2	1·29	0·59	80	75	75	0·46
		5	Second	5·1	1·85	0·84	17·3	20·2	20	0·45
		5	Third	1·5	1·48	0·75	4·2	4·6	4·9	0·51

Table 2. *Separation of the enzymes*

Fraction	Volume (ml.)	Protein (mg./ml.)	Protein (Total mg.)	Glucose 6-phosphate dehydrogenase (Units/mg.*)	Glucose 6-phosphate dehydrogenase (Total units)	Phosphogluconate dehydrogenase (Units/mg.)	Phosphogluconate dehydrogenase (Total units)	Ratio: Phosphogluconate dehydrogenase / Glucose 6-phosphate dehydrogenase
Esch. coli extracts† (*O*)	30	16·4	490	0·274	138	0·109	54	0·40
Supernatant fluid (*S*) from Mn^{2+} pptn.	60	4·18	250	0·460	116	0·188	46·3	0·41
50% $(NH_4)_2SO_4$ ppt. (*A*)	3·5	5·80	20·3	0·161	3·2	0·005	0·1	0·03
60% $(NH_4)_2SO_4$ ppt. (*B*)	3·3	20·3	67·0	1·00	62·4	0·021	1·4	0·021
60% $(NH_4)_2SO_4$ reprecipitate (*C*)	3·3	16·1	53·0	0·92	49·0	<0·014	<0·74	<0·015
62% $(NH_4)_2SO_4$ ppt. (*D*)	3·3	6·06	20·0	1·00	20·0	0·03	0·65	0·03
pH 5·0 ppt. (*E*)	5·5	7·84	43·0	0·267	11·4	0·48	20·8	1·8
pH 4·4 ppt. (*F*)	2·2	3·14	6·9	0·055	0·4	1·30	8·8	23·6
Sum of precipitates			157·2		97·4		31·6	

* A unit of enzyme reduces 1 μmole TPN/min. at 36°.

† Cells extracted twice with 5 vol. of 0·1 M-Tris, pH 7·4.

reactivated by incubation at 4° with glucose 6-phosphate. Freeze-dried dialysed enzyme could be similarly dissolved and reactivated (Table 3).

Phosphogluconate dehydrogenase. This was prepared from the supernatant fluid of the first 60% saturated $(NH_4)_2SO_4$ solution (*B*). The precipitate (*D*) formed at 62–64% saturation was discarded since it contained only a small amount of the desired enzyme and residual glucose 6-phosphate dehydrogenase (Table 2). At this point, several methods were tried for obtaining good yields of a purer phosphogluconate enzyme.

Precipitation by organic solvents at low temperature by the methods of Askonas (1951) resulted in 70% loss of activity. With acetone, enzyme activity was spread over the precipitates throughout the whole range of acetone concentration. Addition of ethanol to dialysed 60% $(NH_4)_2SO_4$ supernatant fluid at pH 6·35 gave a precipitate between 37 and 54% ethanol which contained 70% of the recoverable activity, that is, 27% of the original activity. This step produced a 10-fold increase in phosphogluconate dehydrogenase activity/mg. of protein. The precipitate was dissolved in 0·2M-glycylglycine buffer at pH 7·4 and dialysed immediately against cold running distilled water with no further loss of activity.

A second method, precipitation (*F*) of the enzyme between pH 5·1 and 4·4 from 63% saturated $(NH_4)_2SO_4$ supernatant fluid, produced an 8- to 12-fold increase of activity on phosphogluconate and greatly reduced the glucose 6-phosphate dehydrogenase. The precipitate at pH 5·0 (*E*) still contained appreciable amounts of the latter enzyme.

The third method was saturation with $(NH_4)_2SO_4$. The precipitate (*G*) had 5 times the activity/mg. protein of the original extract and the yield was 56%.

Dialysis of these precipitates (*F* and *G*) dissolved in glycylglycine or water caused precipitation of the enzyme, which was then centrifuged and redissolved in buffer.

RESULTS

Variation of activity with concentration of enzyme

Glucose 6-phosphate dehydrogenase activity was proportional to the amount of enzyme over as wide a range as can be read in the instrument, if the proper precautions were taken in diluting the enzyme solutions. Dilution with water or buffer solution caused a decrease in activity but dilutions with buffer containing substrate maintained proportionality of activity (Table 4).

The rate of reduction of TPN by phosphogluconate dehydrogenase was also proportional to the amount of the enzyme (Fig. 1). However, dilution of the extracts was observed to increase the activity. This increase was most marked in glycylglycine buffer or albumin and was not affected by the presence or absence of substrate. This effect may be due to the dilution of an inhibitor. Therefore, phosphogluconate dehydrogenase activity of ex-

Table 3. *Inactivation and reactivation of glucose 6-phosphate dehydrogenase*

	Activity (ΔE/mg./min.)					
Period at 4°	0	30 min.	60 min.	24 hr.	1 week	3 weeks
(1) Reprecipitated 60% $(NH_4)_2SO_4$ ppt. (*C*)	4·40	—	—	—	—	—
(2) The same, dialysed	1·32	—	—	1·30	2·20	—
(3) Dialysed, freeze-dried resuspended in						
(*a*) water	0·65	—	—	0·85	1·05	—
(*b*) Tris, 0·2M pH 8·0	1·08	—	—	3·75	—	—
(*c*) Tris + G 6-P	1·68	2·79	3·21	3·00	2·73	2·20
(*d*) Tris + G 6-P + Mg	1·38	2·40	—	2·70	2·82	2·55
(*e*) glycylglycine pH 7·4 + G 6-P	—	—	—	2·1	2·32	0·45
(*f*) albumin	—	—	—	1·23	0·52	0

Table 4. *Effect of dilution of extracts*

Extract	Dilution	Diluent	Substrate	Activity (ΔE/mg. prot./min.)
Water extract 1	0	—	Glucose 6-phosphate	1·08
	100	Tris, 0·2M, pH 8·7		0·81
Water extract 2	0	—		1·68
	10	Glycylglycine, 0·2M, pH 7·4		1·09
Water extract 3	0	—	Phosphogluconate	0·75
	10	Glycylglycine, 0·2M, pH 7·4		0·80
	100	Glycylglycine, 0·2M, pH 7·4		0·94
	100	Water		0·78
Water extract 4	0	—		0·40
	10	Glycylglycine, 0·2M, pH 7·4		0·87
Glycylglycine extract 5	0	—	Glucose 6-phosphate	1·24
	5	Tris, 0·2M, pH 8·7 + G 6-P + Mg^{++}		1·72
Tris, pH 7·4, extract 6	0	—		1·82
	5	Tris, 0·2M, pH 8·7 + G 6-P + Mg^{++}		1·85

tracts was measured with samples diluted as much as was practicable.

For maintenance of activity, Tris buffer was best for glucose 6-phosphate dehydrogenase. This enzyme in Tris, pH 8·0, lost only 7 % of its activity after 6 weeks at 4°. Phosphogluconate dehydrogenase did not maintain its activity as well and was more stable in glycylglycine buffer than in Tris. In Tris at pH 8·0, two-thirds of the activity was lost in 6 weeks while in glycylglycine buffer at pH 7·4, one-third was lost in 10 weeks at 4°.

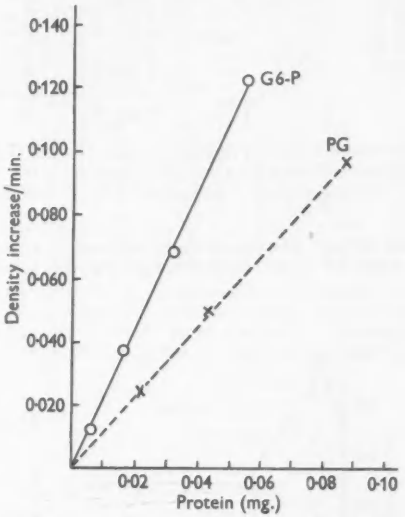

Fig. 1. Concentration of enzyme. G 6-P: Reaction mixtures contained 1 μmole G 6-P, 0·15 μmole TPN, 0·02 M-Mg and enzyme (ppt. *B*, Table 2, dialysed) in 0·04 M glycylglycine at pH 7·4. Total vol. 1 ml. PG: reaction mixtures contained 1 μmole PG, 0·15 μmole TPN, 0·02 M-Ca^{++} and enzyme (ppt. *G*, dialysed) in 0·04 M glycylglycine at pH 7·4. Total vol. 1 ml.

Effect of ions on enzyme activities

Dialysed glucose 6-phosphate dehydrogenase prepared from *Esch. coli* was inactive without the addition of divalent cations. The dehydrogenase from yeast (zwischenferment) prepared by the method of Kornberg (1950) had 33 % of its maximum activity without the addition of any ions. Adsorption on tricalcium phosphate and elution must have left residual calcium ions attached to the enzyme which were not removed by subsequent dialysis. Dialysed phosphogluconate dehydrogenase from *Esch. coli* always had some activity in the absence of any salts except those present in the buffer and substrate.

Both enzymes were activated by bivalent cations as chlorides (see Figs. 2 and 3). While magnesium ions produced maximal activity at concentrations of 0·015 and 0·02 M, calcium ions caused greater activation at a slightly higher concentration and were active over a wider range (Figs. 4 and 5). As will be discussed below, in experiments with varying TPN concentration, magnesium and calcium ions must differ somewhat in their mode of activation. The activating cation must be present in the buffer mixture when the enzyme is added. The order of addition of the other component was without effect on the rate of reduction of TPN.

Table 5. *Inhibition of enzymes by bivalent cations*

Cation	Molarity	Glucose 6-phosphate* dehydrogenase inhibition (%)	Phospho-gluconate† dehydrogenase inhibition (%)
Cupric	2×10^{-3}	90‡	100‡
	1×10^{-3}	75	75
	1×10^{-4}	7	—
Zinc	1×10^{-3}	100‡	75
	1×10^{-4}	93	28
Cadmium	1×10^{-3}	30	72
	1×10^{-4}	—	45
Cobaltic	1×10^{-2}	—	57
	1×10^{-3}	0‡	28
Beryllium	1×10^{-3}	0‡	44
Manganous	1×10^{-2}	—	10
Mercuric	2×10^{-3}	—	84
	1×10^{-3}	100	—
	1×10^{-4}	75	—

* Reaction mixture for glucose 6-phosphate contained 0·018 mg. dialysed enzyme, 0·04 M Tris, pH 8·7, 0·015 M-$MgCl_2$, 2·5 μmole G 6-P and 0·3 μmole TPN.

† Reaction mixture for phosphogluconate contained 0·043 mg. dialysed 100% saturated $(NH_4)_2SO_4$ ppt., 0·04 M-glycylglycine, pH 7·4, 0·02 M-$CaCl_2$, 1 μmole PG and 0·15 μmole TPN.

‡ Denotes precipitation.

Univalent cations, as chlorides at 10 times the concentration of the bivalent ions, could activate phosphogluconate dehydrogenase but not glucose 6-phosphate dehydrogenase (Fig. 6). However, both enzymes in extracts of eggs of the polychaete, *Chaetopterus*, were activated by 0·15 M-sodium chloride or potassium chloride (Cohen, 1951*c*).

Sodium phosphate, bicarbonate and citrate activated glucose 6-phosphate dehydrogenase to about 10 % of the activation with calcium or magnesium ions, but were inhibitory in the presence of calcium or magnesium ions. Sodium sulphate and phosphate activated phosphogluconate dehydrogenase to the extent of 88 and 65 % of the activation by calcium ions, and were inhibitory in the presence of calcium ions only in high concentrations.

In systems containing calcium or magnesium ions the addition of other bivalent cations, copper, cadmium and zinc especially, caused marked inhibition (Table 5). Inhibition was less with

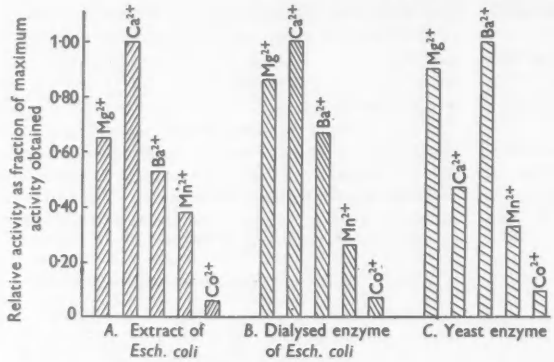

Fig. 2.

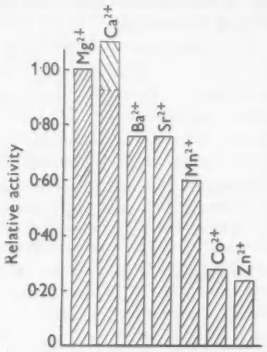

Fig. 3.

Fig. 2. Activation of glucose 6-phosphate dehydrogenase by bivalent cations. Reaction mixtures contained 1 μmole G 6-P, 0·15 μmole TPN, and 0·02 M-salt as chloride in 0·04 M-glycylglycine buffer at pH 7·4 and 36° in total vol. 1 ml., and enzyme as follows: *A*, 0·02 ml. of *Esch. coli* extract (*O*). *B*, 0·016 mg. protein of dialysed ppt. (*B*). *C*, 0·0075 mg. protein of yeast 'zwischenferment'.

Fig. 3. Activation of phosphogluconate dehydrogenase by bivalent cations. Reaction mixtures contained 1 μmole PG, 0·15 μmole TPN, 0·039 mg. protein of dialysed ppt. (*G*) and 0·02 M-salt as chloride in 0·04 M-glycylglycine buffer at pH 7·4 and 36°.

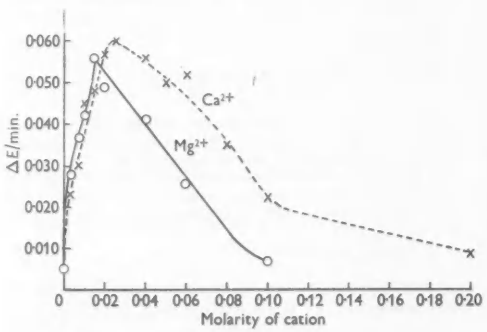

Fig. 4.

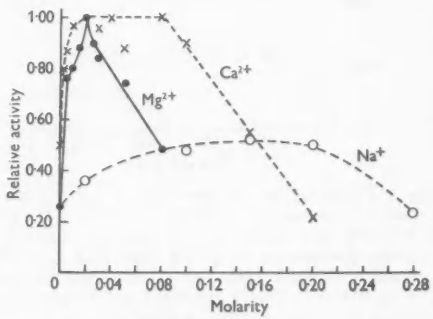

Fig. 5.

Fig. 4. Activation of glucose 6-phosphate dehydrogenase by calcium and magnesium. The reaction mixtures contained 2 μmole glucose 6-phosphate, 0·15 μmole TPN, 0·04 M-Tris, pH 8·17 and 0·06 mg. enzyme in 1 ml. total vol. at 36°. The enzyme was ppt. (*C*), dialysed, freeze-dried, and dissolved in albumin solution.

Fig. 5. Activation of phosphogluconate dehydrogenase by calcium, magnesium and sodium. The reaction mixtures contained 0·72 μmole PG, 0·15 μmole TPN and 0·044 mg. protein of ppt. (*F*), dialysed, in 0·04 M-glycylglycine buffer pH 7·4 at 36°.

extracts than with purified enzymes, owing to the higher concentration of protein in the extracts, which removed these ions from solution. The buffer used also removed these ions. Zinc ions, however, precipitated the proteins. Inhibition of phosphogluconate dehydrogenase by copper sulphate was also noted by Horecker & Smyrniotis (1951).

The effect of variation of concentration of substrate

The activity of partially purified enzymes at various substrate concentrations is shown in Figs. 7 and 8. The Michaelis constant for glucose 6-phosphate combination with enzyme was of the order of $3–5 \times 10^{-4}$. For the phosphogluconate-enzyme complex K_m was $2·2 \times 10^{-5}$ in the presence

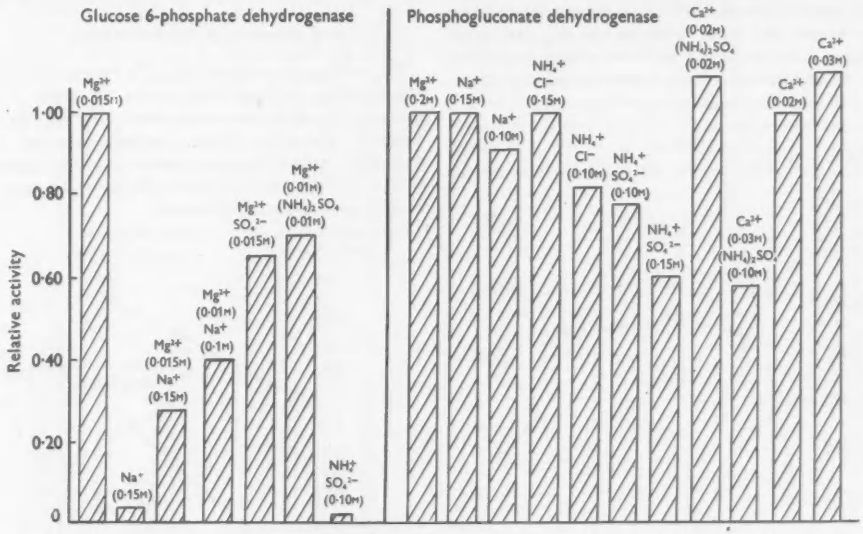

Fig. 6. Effect of sodium, ammonium and sulphate ions on enzyme activity. Glucose 6-phosphate dehydrogenase: the reaction mixtures contained 1 μmole G 6-P, 0·2 μmole TPN, and 0·016 mg. protein of dialysed ppt. (*C*) in 0·04 M-glycylglycine buffer at pH 7·4 and 36°. Phosphogluconate dehydrogenase: the reaction mixtures contained 1 μmole PG, 0·15 μmole TPN and 0·045 mg. protein of dialysed ppt. (*G*) in 0·04 M-glycylglycine buffer at pH 7·4 and 36°.

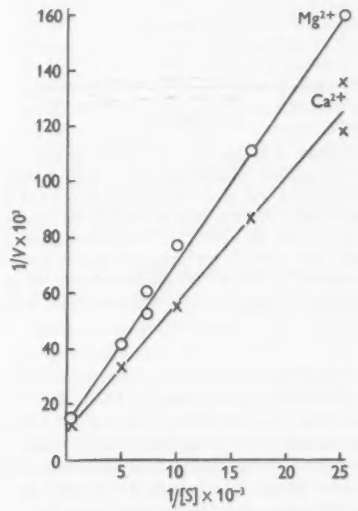

Fig. 7.

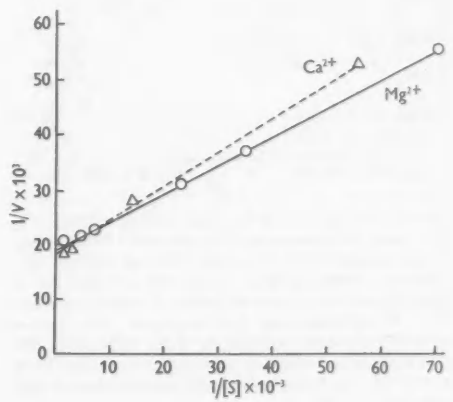

Fig. 8.

Fig. 7. Effect of substrate concentration, glucose 6-phosphate. The reaction mixtures contained 0·3 μmole TPN, 0·032 mg. protein of ppt. (*C*), 0·015 M-$MgCl_2$ or 0·025 M-$CaCl_2$ in 0·04 M-Tris at pH 8·7 and 36°.

Fig. 8. Effect of substrate concentration, phosphogluconate. The reaction mixtures contained 0·15 μmole TPN, 0·044 mg. protein of dialysed ppt. (*F*), 0·02 M-$MgCl_2$ or 0·03 M-$CaCl_2$ in 0·04 M-glycylglycine at pH 7·4 and 36°. K_m, $Mg^{2+} = 2{\cdot}5 \times 10^{-5}$; K_m, $Ca^{2+} = 3{\cdot}3 \times 10^{-5}$.

of magnesium ions and $3{\cdot}3 \times 10^{-5}$ with calcium ions. These are of the same order as the K_m given by Horecker & Smyrniotis (1951). It would seem that magnesium ions hold the substrate more tightly to the latter enzyme than calcium ions.

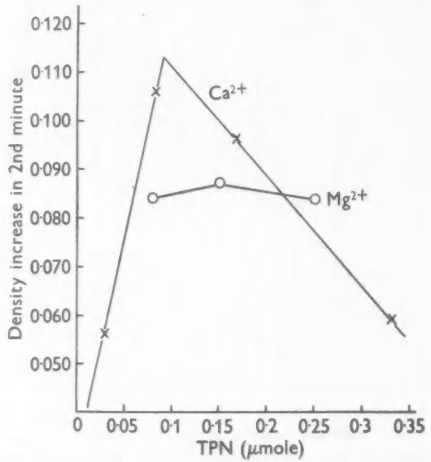

Fig. 9. Effect of TPN concentration on phosphogluconate dehydrogenase. The reaction mixtures contained 1 μmole PG, 0·086 mg. protein of dialysed ppt. (*G*), 0·02 M-$MgCl_2$ or 0·02 M-$CaCl_2$ in 0·04 M-glycylglycine at pH 7·4 and 36°.

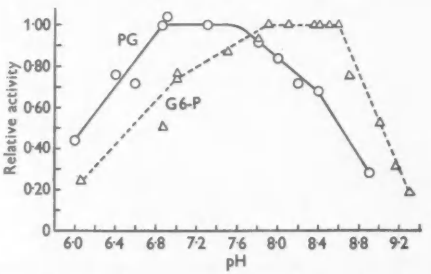

Fig. 10. Effect of pH on enzyme activity. Glucose 6-phosphate dehydrogenase: the reaction mixtures contained 1 μmole G 6-P, 0·3 μmole TPN, 0·01 M-$MgCl_2$ and 0·032 mg. protein of dialysed ppt. (*B*) in 0·04 M-Tris or glycylglycine or mixtures of buffers at indicated pH and 36°. Phosphogluconate dehydrogenase: the reaction mixtures contained 0·15 μmole TPN, 0·36 μmole PG, 0·02 M-$MgCl_2$ and 0·038 mg. protein of dialysed ppt. (*G*) in 0·04 M-Tris or glycylglycine buffer or mixtures, at indicated pH and 36°.

These enzymes may be used for assay of substrate concentration. Separated and partially purified enzymes in amounts sufficient to oxidize all the substrate within 20 min., and 0·4 μmole TPN are necessary for assay of either substrate.

Effect of variation of concentration of TPN

The effect of variation of TPN concentration on the activity of these enzymes is not easily interpreted. Dissociation constants are only approximations, for the state of the enzymes, whether fully activated or not, the ions present, and the presence of non-specific proteins, have effects on the enzyme-coenzyme complex formation.

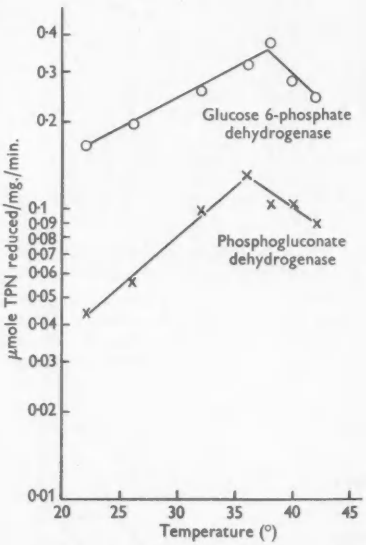

Fig. 11. The effect of temperature on enzyme activity. Glucose 6-phosphate dehydrogenase: the reaction mixtures contained 0·3 μmole TPN, 0·76 μmole G 6-P, 0·015 M-$MgCl_2$ and 0·022 mg. protein of dialysed ppt. (*B*) in 0·04 M-Tris buffer at pH 7·8. Phosphogluconate dehydrogenase: the reaction mixtures contained 0·3 μmole TPN, 0·36 μmole PG, 0·02 M-$MgCl_2$ and 0·034 or 0·068 mg. protein of dialysed ppt. (*G*) in 0·04 M-glycylglycine buffer at pH 7·2.

Glucose 6-phosphate dehydrogenase in extracts had a K_m for TPN of about 4×10^{-5}. For the dialysed enzyme K_m for TPN was 3×10^{-4} with calcium ions and 2×10^{-4} with magnesium ions.

The difference between the action of calcium and magnesium was much more marked with phosphogluconate dehydrogenase (see Fig. 9). The dialysed enzyme reached a peak of activity at 8×10^{-5} M-TPN and fell rapidly as the concentration was increased above this, if calcium was the activating ion. In the presence of 0·02 M-magnesium, however, activity was constant over a range of TPN concentration from 8×10^{-5} to 2×10^{-4} M.

Effect of pH

From Fig. 10, it can be seen that glucose 6-phosphate dehydrogenase was optimally active from pH 7·7 to 8·6, and phosphogluconate dehydrogenase from pH 6·6 to 7·7. The medium in which the bacteria were grown had pH 7·8, at which pH both enzymes have within 5–10 % of their maximum activity.

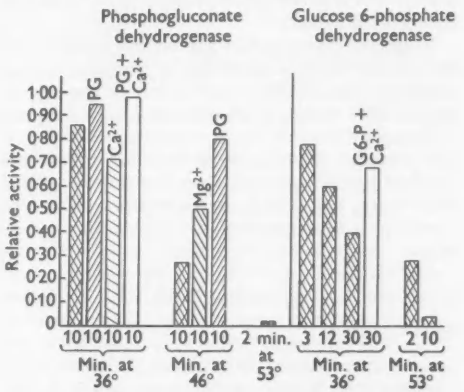

Fig. 12. Heat inactivation of enzymes and protection by substrate. Glucose 6-phosphate dehydrogenase: the reaction mixtures contained 2 μmole G 6-P, 0·15 μmole TPN, 0·025 M-$CaCl_2$ in 0·04 M-Tris buffer at pH 8·7 at 36°. The enzyme was 0·06 mg. freeze-dried, dialysed, ppt. (*C*), dissolved in 0·06 % serum albumin and held at indicated temperatures for varying lengths of time. Phosphogluconate dehydrogenase: the reaction mixtures contained 0·2 μmole TPN, 0·70 μmole PG, 0·03 M-$CaCl_2$ in 0·04 M-glycylglycine buffer pH 7·4, at 36°. The enzyme was the precipitate formed on dialysis of ppt. (*G*) dissolved in glycylglycine buffer pH 7·4. The enzyme was held at temperatures indicated for indicated times with or without the presence of substrate or salts.

Effect of temperature

Activation. As shown in Fig. 11, both enzymes had maximal activity at 37°. Between 22 and 36°, glucose 6-phosphate dehydrogenase had a temperature coefficient of 1·55 and phosphogluconate dehydrogenase, 2·2. At higher temperatures inactivation of the enzymes occurred rapidly.

Inactivation. Extracts and partially purified enzymes lost activity rapidly when unprotected by the substrate. Glucose 6-phosphate dehydrogenase was 40 % inactivated in 12 min. at 37° and about 10 % inactivated in 24 hr. at 4°. Phosphogluconate dehydrogenase lost 15 % of its activity in 12 min. at 37°. At 53° for 2 min., almost all phosphogluconate activity was lost, but 30 % of glucose 6-phosphate activity remained. However, the enzyme could be protected very markedly by substrates, by buffer or albumin solutions somewhat, and by magnesium ions, but not by calcium ions. In the reaction mixture, complete except for TPN, both enzymes were stable at all temperatures up to 36° (Fig. 12).

The enzymes were not protected by TPN, but rather the activity of both enzymes was decreased about 50 % by 5×10^{-4} M-TPN added to dilutions of the extract in Tris. Separated phosphogluconate dehydrogenase was precipitated from glycylglycine solution by $2{\cdot}8 \times 10^{-3}$ M-TPN and could not be redissolved or reactivated.

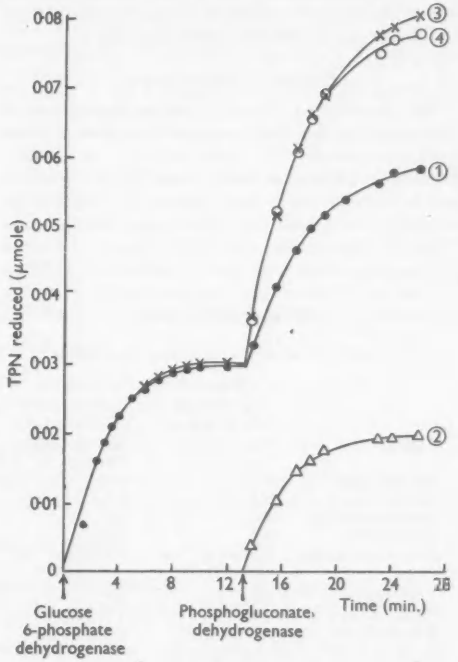

Fig. 13. The reaction mixtures contained 0·025 M-$CaCl_2$ and 0·20 mg. protein of ppt. (*C*) in 0·04 M-glycylglycine at pH 7·4. Cuvette (1) contained 0·03 μmole G 6-P. Cuvette (2) contained 0·0196 μmole PG. Cuvette (3) contained 0·03 μmole G 6-P + 0·0196 μmole PG. At zero time, 0·28 μmole TPN was added. At 13 min. 0·18 mg. of phosphogluconate dehydrogenase, ppt. (*F*), was added. ●—●, 0·030 μmole G 6-P; △—△, 0·0196 μmole PG; ×—×, 0·030 μmole G 6-P + 0·0196 μmole PG; ○—○, the sum of curves (1) and (2).

Oxidation by phosphogluconate dehydrogenase of the product of glucose 6-phosphate dehydrogenase

Fig. 13 shows the action of the separated enzymes. The glucose 6-phosphate dehydrogenase caused no reduction of TPN in the presence of phosphoglu-

conate. Phosphogluconate dehydrogenase was equally unreactive in the presence of glucose 6-phosphate. The latter enzyme reduced the phosphogluconate, which had been produced by the first enzyme, at the same rate as it reduced synthetic phosphogluconate. This would seem to indicate that, if the primary product of the glucose 6-phosphate dehydrogenase action is the phosphogluconolactone (Cori & Lipmann, 1952), either there was a lactonase present in one or both of the enzyme preparations, or the lactone was hydrolysed chemically.

Methods of enzyme assay

By selecting the proper conditions, the activity of either enzyme can be determined in extracts where both are present. The order of addition of components is immaterial except that the bivalent ion and buffer must be in the mixture when the enzyme is added. The chosen methods of assay are shown in Table 6. Under these conditions of assay of glucose 6-phosphate dehydrogenase, reduction of TPN by the action of the other enzyme on the phosphogluconate produced was minimized.

Table 6. *Conditions for assay of enzymes*

	Glucose 6-phosphate dehydrogenase	Phospho-gluconate dehydrogenase
Buffer	Tris, 0·04 M	Glycylglycine, 0·04 M
pH of buffer	8·7	7·4
pH of mixture	8·5	7·2
μmole substrate	2·0	0·7
μmole TPN	0·3	0·15
Salt concentration	0·015 Mg^{++} or 0·025 Ca^{++}	0·020 Mg^{++} or 0·025 Ca^{++}
Temperature	36°	36°
Total volume	1 ml.	1 ml.
Enzyme*	0·01–0·1 ml.	0·01–0·1 ml.

* Cf. Table 1.

These conditions for assay of glucose 6-phosphate dehydrogenase are quite different from those given by Kornberg (1950) for zwischenferment. Values obtained by the latter method may be multiplied by a factor of 2·4 to give values corresponding to those obtained by the method in Table 6 at 26°. Including the temperature coefficient, the factor is 3·9 for assay at 36°.

Our conditions for assay of phosphogluconate dehydrogenase are, except for temperature, approximately the same as those employed by Horecker & Smyrniotis (1951) and Seegmiller & Horecker (1951), and give the same values for activity of enzymes.

DISCUSSION

The two dehydrogenases of the oxidative pathway of glucose 6-phosphate obtained from extracts of *Esch. coli* have been separated and partially purified. Both enzymes were comparatively labile when separated from each other and from some of the other bacterial protein, especially in dilute solution. They could, however, be stabilized by mixture with buffer or non-specific protein, or even better by the presence of substrate and magnesium ion in the buffer. Both enzymes were specific for reduction of TPN and had no action with DPN. Both enzymes were activated by calcium and magnesium though the effect of different concentration of the ions was somewhat different.

In other properties, the two enzymes differed. The pH optima and the temperature coefficients were different. The greater concentration of substrate and of TPN needed for the activity of the glucose 6-phosphate enzyme and the greater sensitivity of this enzyme to inbibition by ions other than the bivalent-cation chlorides are noteworthy. On the other hand, the phosphogluconate enzyme showed a sensitivity to concentration of TPN not so evident in the former enzyme. Concentrations of TPN above 1×10^{-4} M were inhibitory to the reaction, and incubation of the enzyme with TPN in higher concentration may inactivate the enzyme entirely.

Whether these differences in specificity and sensitivity reflect the natural state of the enzyme in the intact cell it is impossible to say at the present time. It might be that conditions of temperature, pH, TPN and ion concentration in the cell determine what part of the metabolism of glucose 6-phosphate is to be carried over the oxidative route and what part over the glycolytic route. The phosphogluconate dehydrogenase was active under a wide variety of environmental conditions.

SUMMARY

1. Glucose 6-phosphate dehydrogenase and phosphogluconate dehydrogenase were obtained from extracts of *Escherichia coli*, and were separated and partially purified.

2. Optimal conditions for the action of these enzymes in reducing triphosphopyridine nucleotide in the presence of their substrates and for their assay were determined.

3. Both enzymes were activated by bivalent cations as chlorides. Magnesium and calcium ions were most active at about 2×10^{-2} M concentration.

4. Michaelis constants for the enzyme-substrate complex were of the order of 3×10^{-4} for glucose 6-phosphate dehydrogenase and 3×10^{-5} for phosphogluconate dehydrogenase.

5. Optimal pH was 7·7–8·6 for glucose 6-phosphate dehydrogenase and 6·6–7·7 for phosphogluconate dehydrogenase, and maximal activity of both enzymes occurred at 37°.

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The Oxidative Pathway of Carbohydrate Metabolism in *Escherichia coli*

2. QUANTITATIVE STUDIES OF GLUCOSE 6-PHOSPHATE DEHYDROGENASE AND 6-PHOSPHOGLUCONATE DEHYDROGENASE

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In this paper data are presented on the activities of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase extracted from *Escherichia coli* in an effort to estimate the proportion of carbohydrate which might be metabolized by the enzymes during bacterial growth under aerobic conditions. Enzymes were extracted which accounted for approximately 40 % of the total carbohydrate metabolized by the cell.

METHODS

As will be shown, the amounts of these enzymes in *Esch. coli* varied according to the conditions of growth. The following procedure was followed in all experiments presented in this paper. *Esch. coli*, strain B, from the stock culture in synthetic medium (Cohen, 1949), was used to inoculate 100 ml. of synthetic medium containing 1 mg. glucose/ml. After 17 hr. aerobic growth at 36° the glucose was exhausted and the culture contained 9×10^8 cells/ml.; 50 ml. were then inoculated into each of two roller flasks containing 250 ml. of synthetic media with the desired concentration of carbohydrate as sole carbon source. The flasks were rotated at 300–400 rev./min. in the incubator at 36°. While spinning at this rate the fluid formed a band completely around the flask, thus assuring maximal aeration of the culture. The growth of the culture was followed by measurement of the turbidity at 420 mμ. in the Klett photometer and sometimes by plate count. Fig. 1 shows the correlation of turbidity with plate count during growth of the bacteria on glucose or potassium gluconate.

The cells were harvested either during exponential growth or when the carbon source, or the nitrogen source, or both, were exhausted. The culture was immediately chilled to 4°. Centrifugation, washing, and extraction were carried out at 2–4°. The bacteria were disrupted by grinding with alumina (McIlwain, 1948) and extracted two or three times with water or tris(hydroxymethyl)aminomethane buffer (Tris) at pH 8·0. Determination of the enzyme activities was made by measurement of the reduction of triphosphopyridine nucleotide (TPN) in the presence of glucose 6-phosphate (G 6-P) or 6-phosphogluconate (PG) by the methods described in the preceding paper (Scott & Cohen, 1953).

RESULTS

At the beginning of growth on glucose in the roller flask there was either no lag phase or it was very short. With gluconate there was usually a lag of about 20–40 min., presumably for formation of the adaptive enzyme, gluconokinase (Cohen & Scott, 1950; Cohen, 1951*a*).

With 1 mg. glucose/ml. in the medium the bacteria exhausted the substrate when the turbidity was 190 and the count 9×10^8 cells/ml. With greater concentrations of glucose, growth, that is increase in turbidity and in cell count, was exponential and the extent of growth was proportional to the initial glucose concentration up to the limit of the nitrogen source in the medium (Monod, 1942). The medium contained 21·2 mg. N/ml. as ammonium sulphate and with excess glucose allowed exponential growth up to populations of 3×10^9 cells/ml. At this point, the cells continued to divide at least once, although the rate of increase in turbidity fell off to zero.

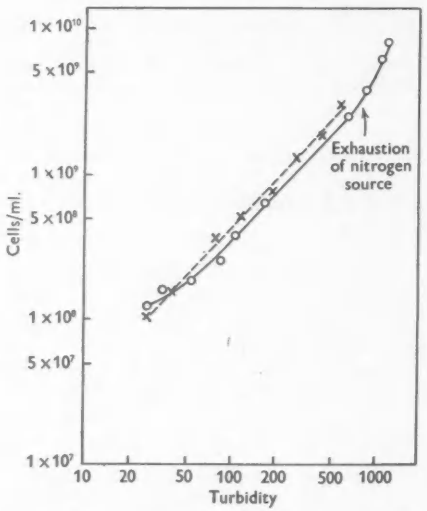

Fig. 1. Correlation of turbidity with plate count during growth of *Esch. coli* in glucose or gluconate. O—O, 10 mg./ml. glucose; ×—×, 11 mg./ml. potassium gluconate.

The mass and cell doubling time during the exponential phase of growth was 45–55 min. with an average of 50 min. on either glucose or gluconate. However, it can be seen in Fig. 1 that cultures grown in gluconate may have 20 % lower turbidity than those prepared in glucose, although both may contain the same numbers of cells.

The substrate consumption per cell is constant during growth, i.e. both are exponential (Monod, 1942). Therefore, during the last 50 min. of exponential growth, the bacteria have doubled in turbidity and plate count, and have used half the available substrate. The number of molecules of carbohydrate used/cell/min. was determined by the moles of glucose or gluconate used/ml./min. × $6{\cdot}06 \times 10^{23}$ and divided by the number of cells/ml. Table 1 gives the values calculated from turbidity and plate counts which were determined experimentally. The mean values were $8{\cdot}5 \times 10^7$ molecules of glucose/cell/min. or $6{\cdot}3 \times 10^7$ molecules of potassium gluconate/cell/min.

In the extracts prepared from the cells the total activities of both glucose 6-phosphate and phosphogluconate dehydrogenase were determined in terms of moles of substrate oxidized/min. This value, multiplied by $6{\cdot}06 \times 10^{23}$ and divided by the total number of cells from which the extract was prepared, gave the number of molecules of substrate oxidized/cell/min.

Data given in Table 1 show the following:

(1) Extracts from cells harvested during the exponential phase on glucose contained enzymes sufficient to account for oxidation at the rate of $2{\cdot}6$–$6{\cdot}9 \times 10^7$ molecules of glucose 6-phosphate/cell/min., and $2{\cdot}0$–$3{\cdot}7 \times 10^7$ molecules of phosphogluconate/cell/min. As was noted previously (Scott & Cohen, 1953), the lower values were usually obtained with a water extract. Later experiments in which the disrupted cells were extracted with Tris gave values of $6{\cdot}0$–$6{\cdot}9 \times 10^7$ molecules of glucose 6-phosphate/cell/min.

(2) Cells which had exhausted their nitrogen source but not their glucose, and were still dividing, were smaller but had a high content of enzymes, especially of glucose 6-phosphate dehydrogenase.

(3) In extracts of cells which had ceased to grow because of exhaustion of glucose or gluconate the activities of both enzymes were low.

(4) When gluconate was the carbon source, extracts made from cells harvested during the exponential phase of growth contained enzymes sufficient to account for oxidation at the rate of $2{\cdot}2$–$5{\cdot}2 \times 10^7$ molecules of glucose 6-phosphate/cell/min. and $1{\cdot}5$–$2{\cdot}6 \times 10^7$ molecules of phosphogluconate/cell/min.

DISCUSSION

The apparent content of glucose 6-phosphate dehydrogenase in cells harvested during exponential growth on glucose could account for 31–86 % of the glucose metabolized as glucose 6-phosphate. But the extractable activity of phosphogluconate dehydrogenase accounted for a maximum of 44 % of the glucose metabolized. The difference between the apparent amounts of the two dehydrogenases in the cell may have one or more of several explanations.

(1) Since the activity of glucose 6-phosphate dehydrogenase is so extremely sensitive to factors of dilution, of protein concentration, of ion concentration and especially of substrate concentration, perhaps our optimal conditions of assay represent more nearly the maximum capability of the enzyme rather than the functioning activity of the cell *in vivo*. The pH should not be considered a factor here,

Table 1. *Correlation of metabolic and enzymic activities*

Substrate (mg./ml.)	Growth phase when harvested	Turbidity	Extractant	Cells: Molecules substrate used/cell/min. $\times 10^7$	Extracted enzymes: Molecules G 6-P† oxidized/cell/min. $\times 10^7$	Extracted enzymes: Amount of substrate accounted for (%)	Extracted enzymes: Molecules PG‡ oxidized/cell/min. $\times 10^7$	Extracted enzymes: Amount of substrate accounted for (%)
				Glucose				
2	Exponential	310	Water	8·3	4·5	54	2·8	34
5	Exponential	510	Water	8·5	3·6	41	3·1	37
10	Exponential	635	Water	8·4	2·6	31	2·0	24
10	Exponential	640	Water	8·0	4·0	50	2·1	26
10	Exponential	600	Water	9·2	4·3	47	3·8	41
10	Exponential	475	Tris	8·0	6·9	86	3·2	40
5	Exponential	515	Tris	8·2	6·1	74	3·7	44
10	Exhaustion of N	750	Water	7·8*	6·2	56	3·0	38
5	Exhaustion of N	800	Tris	8·2*	6·0	73	3·1	38
2	Exhaustion of substrate	435	Water	8·3*	2·2	—	1·5	—
5	Exhaustion of N and substrate	776	Water	8·5*	1·3	—	1·3	—
				Gluconate				
2·7	Exponential	320	Water	5·3	2·2	41	1·5	28
6·0	Exponential	395	Water	6·6	2·7	41	1·8	27
11·4	Exhaustion of N	650	Water	6·2*	2·4	39	1·5	24
6·0	Exponential	585	Tris	7·2	5·2	72	2·6	36
2·5	Exhaustion of substrate	345	Water	5·8*	2·1	—	1·1	—
2·0	Exhaustion of substrate	260	Water	6·8*	1·1	—	1·2	—

* Calculated during exponential growth. † G 6-P = glucose 6-phosphate. ‡ PG = 6-phosphogluconate.

since at the pH of assay (8·5) the enzyme has within 10 % of the activity at the pH of growth (7·8).

(2) It may be that not all the phosphogluconate which is formed from glucose 6-phosphate is degraded by phosphogluconate dehydrogenase. Perhaps some of the phosphogluconate is split by a mechanism similar to the cleavage by *Pseudomonas saccharophila* into pyruvate and triose phosphate (Entner & Doudoroff, 1952). This reaction has not yet been shown to occur in *Esch. coli*, but according to preliminary experiments, non-oxidative breakdown of phosphogluconate by extracts cannot be ruled out. However, the rate of this breakdown seems to be too slow in the extracts to account for the difference in activity of the two enzymes.

(3) The method of determining glucose 6-phosphate dehydrogenase in extracts may allow a slight reduction of TPN by the phosphogluconate generated. This effect would give falsely high values for glucose 6-phosphate dehydrogenase. This possibility is difficult to rule out, but from experiments with mixtures of the purified enzymes it appears that this effect must be small under the established conditions of assay.

(4) The phosphogluconate dehydrogenase may have been less completely extracted from the cells than the first enzyme or may have suffered greater inactivation during extraction. However, it was noted that the amount of phosphogluconate dehydrogenase obtained in successive extractions of the same cells was proportional to the amount of total protein extracted. Phosphogluconate dehydrogenase is less sensitive to inactivation than glucose 6-phosphate dehydrogenase when conditions are not optimal.

The values for the activity of phosphogluconate dehydrogenase are estimated to account for a mean of 36 % and a maximum of 44 % of the total carbohydrate utilization, which agrees remarkably well with the maximal figure found by the isotope technique (Cohen, 1951*b*) of about 38 % of glucose metabolized via the oxidative pathway. From this it might be inferred that the measurement of the activity of the phosphogluconate dehydrogenase in extracts more nearly represents the enzyme activity in the cell than does the measurement of glucose 6-phosphate dehydrogenase which may merely be present in excess.

Extracts of bacteria grown on potassium gluconate contained less of both enzymes than those grown in glucose. However, these bacteria used less substrate for growth, and the glucose 6-phosphate dehydrogenase extracted accounted for 30–72 %, and the phosphogluconate dehydrogenase for 24–36 % of the substrate metabolized.

Isotope studies showed 85–95 % recovery of the ^{14}C as carbon dioxide, with cells growing on [1-^{14}C]gluconate and 100 % recovery with cells metabolizing [1-^{14}C]gluconate without a nitrogen source for growth (Cohen, 1951*b*). It was considered that the overall reaction:

glucose 6-phosphate → phosphogluconolactone → phosphogluconate

was practically irreversible in intact bacteria under physiological conditions. Glucose dehydrogenase, for example, has been shown to be slowly reversible at pH 3·5 but essentially irreversible at pH 7 (Strecker & Korkes, 1952). Similarly, glucose 6-phosphate is probably not formed from 6-phosphogluconate and reduced TPN by a reversible reaction. It would, nevertheless, be expected that during utilization of gluconate some glucose 6-phosphate would be formed in the cell, arising from condensation products of two-carbon fragments (Dische, 1952; Horecker, 1951; De la Haba & Racker, 1952) or perhaps from triosephosphates derived from the splitting of pentose phosphates or from the cleavage of phosphogluconate (Entner & Doudoroff, 1952). Such a formation of glucose 6-phosphate in the cell metabolizing gluconate may explain the presence of the glucose 6-phosphate dehydrogenase, since the substrate may stimulate the production of the enzyme.

An explanation for a maximal content of phosphogluconate dehydrogenase sufficient to account for only 36 % of the gluconate used despite the 85–100 % recovery of the C—1 of gluconate as carbon dioxide might lie in the adaptive formation of the cleaving system of Entner & Doudoroff (1952). The pyruvate formed by this cleavage contains the C_1 of gluconate in the carboxyl group. Decarboxylation of the pyruvate would produce labelled carbon dioxide.

SUMMARY

1. *Escherichia coli*, strain B, growing on glucose metabolized $8{\cdot}5 \times 10^7$ molecules of glucose/cell/min.

2. Enzymes extracted from cells grown on glucose oxidized glucose 6-phosphate at a rate equivalent to $2{\cdot}6$–$6{\cdot}9 \times 10^7$ molecules/cell/min., and phosphogluconate at a rate equivalent to 2·0–$3{\cdot}8 \times 10^7$ molecules/cell/min.

3. *Esch. coli* growing on potassium gluconate metabolized $6{\cdot}3 \times 10^7$ molecules of gluconate/cell/min.

4. Enzymes extracted from cells grown on potassium gluconate oxidized glucose 6-phosphate at a rate equivalent to $2{\cdot}2$–$5{\cdot}2 \times 10^7$ molecules/cell/min. and phosphogluconate at a rate equivalent to $1{\cdot}5$–$2{\cdot}6 \times 10^7$ molecules/cell/min.

5. These enzymes are still present though decreased in amount in cells which have exhausted their carbon source.

6. Thus enzymes of the oxidative pathway have been extracted from growing cells which account for approximately 40 % of the total metabolism of carbohydrate.

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Observations on the Fate of Some Aliphatic Sulphonic Acids in the Rat

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Detailed studies on the metabolism of sulphonic acids in the animal have been mainly limited to members of the aromatic series (cf. Williams, 1947). The earlier work on the fate of administered aliphatic sulphonic acids is conflicting and has been discussed by Schmidt & Clark (1922). More recent investigations suggest that, with the possible exception of L-cysteic acid, these compounds are not oxidized to any significant extent to sulphate or thiosulphate, as was claimed by Salkowski (1918) and others, but are probably excreted unchanged. However, the reported excretions of various sulphonic acids administered to animals, determined either from increases in urinary neutral sulphur or by direct isolation of salts of the acids from urine, have not been quantitative and do not preclude the possibility of metabolism of these compounds by other paths.

In the experiments described here, the sodium salts of some aliphatic sulphonic acids, related structurally to carboxylic acids of biochemical interest, have been administered to rats by subcutaneous injection, and a study has been made of their effect on the output of urinary sulphate. These acids were methanesulphonic, iodomethanesulphonic, isethionic (β-hydroxyethanesulphonic), β-sulphopropionic and sulphoacetic acids and taurine. None of these compounds was found to be oxidized to sulphate. Changes in the neutral sulphur excretion of dosed animals and the isolation of two of the acids from urine as their phenylhydrazine salts indicate that these six sulphonic acids are excreted largely if not entirely in unchanged form.

MATERIALS AND METHODS

Preparation of compounds. Sodium methanesulphonate and sodium sulphamate were prepared from commercial samples of the acids; sodium iodomethanesulphonate and disodium sulphoacetate were prepared by the action of Na_2SO_3 on iodoform and monochloroacetic acid respectively (Lauer & Langkammerer, 1935; Stillich, 1906). Aminomethanesulphonic acid and α-aminoethanesulphonic acid were obtained from formaldehyde and acetaldehyde respectively by the action of $NaHSO_3$ and NH_3 (Raschig & Prahl, 1926; Backer & Mulder, 1934). Propionic acid was sulphonated photochemically to β-sulphopropionic anhydride which was converted to disodium β-sulphopropionate (Kharasch, Chao & Brown, 1940). Sodium isethionate was prepared by the action of ethylene oxide on $NaHSO_3$ (Lauer & Hill, 1936). The taurine was a commercial preparation (Eastman Kodak Co.). All compounds were free from sulphate and sulphite.

Plan of metabolism experiments. Pairs of male rats (100–150 g. wt.) were housed in metabolism cages with free access to water and to a standard diet of the following percentage composition: starch 34·8, plain flour 25, casein 10, dried yeast 4, margarine 22, cod-liver oil 2, marmite 2, choline chloride 0·2. The urine was collected by means of a large glass funnel and urine-faeces separator, as described previously (Maw, 1948) and preserved under toluene. Each pair of rats was kept in a metabolism cage for an acclimatization period of 4 days. The urine was collected every 24 hr. for the following 4–5 days. The dose of the appropriate compound, neutralized to pH 7 and dissolved in 2 ml. of water, was then injected subcutaneously and the urine collected every 24 hr. for a further period of 4–5 days. Each 24 hr. urine collection was made up to 100 ml. with washings from the funnel and separator, filtered, and inorganic sulphate, total sulphate and total sulphur estimated on 5 ml. samples of the filtrate. In experiments with sodium iodomethanesulphonate, inorganic iodide and iodate and organic iodine were also estimated.

Estimation of sulphate. The method for the estimation of inorganic and total sulphate was essentially that of Rosenheim & Drummond, as modified by Fiske (1921). In preliminary experiments inorganic phosphate was removed prior to the benzidine precipitation, but this was subsequently omitted when it was found that the results of inorganic sulphate estimations carried out with removal of phosphate on fourteen 24 hr. urines selected at random were not significantly different from the corresponding values obtained without removal of phosphate. The P/S ratios of urines from rats fed on the standard diet described above ranged from 1·4 to 3·6. The urines of rats fed on a diet of rat cubes contained, on the other hand, a much higher proportion of phosphate, and sulphate analyses could not be satisfactorily carried out on these urines unless the phosphate was first removed. The sulphonic acids used had no detectable effect on the estimation of sulphate, whether in pure solution or in urine, when present at concentrations which would be expected in the urines collected in the metabolism experiments.

Estimation of total sulphur. This was estimated by Fiske's (1921) method, with slight modifications in the incineration with Benedict's (1909) reagent to avoid loss by spluttering. This method was also used for estimation of sulphur in the sulphonic acids prepared.

Estimation of iodine. Organically bound iodine in urine was determined by a method based on that of Piria & Schiff (Schiff, 1879), the iodate formed being estimated iodometrically. Normal urines by this method gave titres equivalent to those of blank estimations on the reagents alone. Analysis of sodium iodomethanesulphonate by this procedure was quantitative.

RESULTS

Significance of changes in outputs of urinary sulphate and neutral sulphur. Changes occurring in the outputs of the urinary sulphur fractions have been evaluated in the generally accepted manner, by determining the average normal or basal 24 hr. outputs over the pre-dosage period and comparing these values with successive 24 hr. outputs obtained during the period following administration of the compound under study. Provided that rats were given the preliminary acclimatization period of at least 4 days in the metabolism cages, their normal outputs of inorganic and ethereal sulphate and neutral sulphur became steady and reproducible. Table 1 gives complete data for three typical metabolism experiments in which no oxidation of the administered compounds took place. The inorganic and ethereal sulphate values showed relatively small fluctuations from the mean 24 hr. outputs over the whole period. This was also true of the neutral sulphur values during the pre-dosage period, and increases occurring during the experimental period could be determined with an accuracy of about ± 0·5 mg. sulphur. The direct effect of the various sulphonic acids on endogenous sulphur excretion has not been assessed. Neither sodium isethionate nor sodium iodomethanesulphonate, given by mouth or by injection at the dosage levels employed in the metabolism experiments, produced significant changes in food consumption. Subcutaneous injection rather than ingestion was chosen as the mode of administration. While obviating complicating effects due to the action of intestinal bacteria, etc., this route undoubtedly results in rapid renal excretion of the compounds and consequently a reduction in their availability for possible metabolic reactions.

Changes in urinary inorganic sulphate and neutral sulphur following the administration of sulphonic acids to rats. Table 2 summarizes the results of a series of nine experiments in which four sulphonic acids were administered subcutaneously to pairs of rats at a dosage level of approximately 0·85 m-moles/rat. Following the injection of sodium methanesulphonate, sodium iodomethanesulphonate and taurine, no changes in the outputs of inorganic sulphate and ethereal sulphate occurred. Sodium isethionate produced consistent but very slight increases in inorganic sulphate equivalent to 1–3 % of administered sulphur which were not statistically significant. Preliminary experiments with disodium β-sulphopropionate, disodium sulphoacetate and sodium sulphamate have shown that they are also not metabolized to sulphate. Table 2 shows that the administration of the compounds caused a rise in the neutral sulphur fraction of the urine. Except in one experiment with taurine, this increase invariably occurred only during the first 24 hr. period following the dose, and as illustrated in Table 1, the neutral sulphur outputs then returned to normal. The increases taking place over this 24 hr. period amounted to 92–98 % of the sulphur content of the injected doses. Following the administration of sodium iodomethanesulphonate to rats, no iodide or iodate were found in the urine. Furthermore, organically bound iodine, which appeared in the urine only during the 24 hr. period immediately after injection, corresponded to 101–102 % of the dose. By contrast, the recovery of sulphur in the neutral sulphur fraction in experiments with taurine was far from quantitative (58 and 73·3 %). In one experiment, an increased

Table 1. *Outputs of urinary inorganic and ethereal sulphate and neutral sulphur of pairs of rats before and after administration of some aliphatic sodium sulphonates*

(Values are expressed as mg. S/rat/day. The dose of sulphonate, given in parentheses, was injected into each rat on the day indicated.)

Day	1	2	3	4	5	6	7	8	9
Exp. 3. Sodium methanesulphonate (103 mg.) on the 5th day									
Inorganic sulphate	3·86	3·80	3·90	3·71	3·87	3·79	3·63	3·91	
Ethereal sulphate	0·47	0·24	0·35	0·30	0·29	0·26	0·34	0·44	
Neutral sulphur	1·05	1·56	1·46	1·00	1·27	28·53	1·56	1·48	
Exp. 8. Taurine (107 mg.) on the 4th day									
Inorganic sulphate	1·87	2·08	1·72	1·42	1·57	1·82	2·03	2·15	2·00
Ethereal sulphate	0·39	0·43	0·26	0·38	0·40	0·43	0·45	0·45	0·50
Neutral sulphur	0·75	0·75	0·83	0·80	16·58	0·77	0·64	0·89	0·69
Exp. 9. Sodium isethionate (126 mg.) on the 4th day									
Inorganic sulphate	1·65	1·43	1·68	1·58	2·17	2·03	1·62	1·58	
Ethereal sulphate	0·41	0·44	0·48	0·46	0·34	0·36	0·40	0·40	
Neutral sulphur	0·35	0·43	0·31	0·42	26·00	0·30	0·45	0·48	

Table 2. *Changes in the outputs of inorganic sulphate and neutral sulphur of pairs of rats following the administration of some aliphatic sodium sulphonates*

Compound administered	Dose (mg./rat)	Average pre-injection outputs (mg./S/rat/day)		Outputs in 24 hr. after injection (mg./S/rat/day)		Recovery (% of dose)	
		Inorganic sulphate	Neutral sulphur	Inorganic sulphate	Neutral sulphur	Inorganic sulphate	Neutral sulphur
Sodium methanesulphonate	103	3·81	1·34	3·79	28·53	0	97·3
	103	3·54	1·64	3·45	28·16	0	95·0
Sodium iodomethanesulphonate	157	4·63	2·19	4·31	22·43	0	98·3
	151	4·71	2·20	4·31	21·00	0	95·1
Sodium isethionate	125	3·58	1·76	3·94	27·62	(1·3)	95·3
	125	3·28	1·24	4·07	26·17	(2·9)	91·9
	126	1·58	0·39	2·17	26·00	(2·1)	94·1
Taurine (sodium salt)	107	2·56	0·63	2·34	20·80	0	73·3*
	107	1·77	0·78	1·57	16·58	0	58·0

* Increase in neutral sulphur on 2nd and 3rd days after injection = 5·4%. Total recovery = 78·7%.

output corresponding to 5·4%, continued during the 2nd and 3rd days after dosage, giving a total recovery of 78·7%.

Isolation of sulphonic acids from urine. Attempts were made to identify the material causing the increase in the neutral sulphur fraction of the urine after administration of sodium iodomethanesulphonate and sodium methanesulphonate to rats. The urines of eight rats, each of which had been injected 24 hr. previously with 125 mg. of sodium iodomethanesulphonate, were pooled and evaporated to dryness under reduced pressure. The residue was continuously extracted for 3 hr. with 50 ml. of boiling 90% aqueous ethanol and the resulting red solution evaporated to small bulk. This solidified on cooling to a brown crystalline mass, which was washed with absolute ethanol, decolorized with Norite charcoal and recrystallized from 95% aqueous ethanol. The product, which appeared from its elementary analysis to be impure sodium iodomethanesulphonate, was converted into the phenylhydrazine salt (Latimer & Bost, 1937) by dissolving in 5 ml. of water and adding 1 ml. of concentrated H_2SO_4, followed by 2·5 g. of phenylhydrazine in 1 ml. of ethanol. The mixture was cooled in ice and the resulting crystalline mass washed with cold ethanol and ether and recrystallized from absolute ethanol. The melting point of the crystals (166–166·5°) was not depressed by admixture with authentic phenylhydrazine iodomethanesulphonate, m.p. 166–166·5°. (These and other recorded melting points are uncorrected.) (Found: C, 25·5; H, 3·2; N, 8·1; equiv. (by titration) 331. Calc. for $C_7H_{11}O_3N_2SI$: C, 25·5; H, 3·4; N, 8·5%; equiv. 330.) The yield was 1·16 g. (71% recovery of the sulphonic acid administered).

In a similar experiment, in which sodium methanesulphonate was administered to rats at the same dosage level, the phenylhydrazine salt isolated had m.p. 195–195·5°; the mixed melting point with authentic phenylhydrazine methanesulphonate (m.p. 195–196°) was 195–196°. (Found: C, 41·0; H, 5·6; N, 13·6; equiv. (by titration) 204. Calc. for $C_7H_{12}O_3N_2S$: C, 41·2; H, 5·9; N, 13·7%; equiv. 204.) The yield was 1·47 g. (70% recovery of the sulphonic acid administered). The recoveries of the two unchanged sulphonic acids from rat urine thus accounted in each case for about three-quarters of the increased neutral sulphur output. Attempts to isolate the acids as their *S*-benzyl-ψ-thiuronium salts were unsuccessful, owing to the appreciable solubility of these derivatives in water and ethanol.

Experiments with aminomethanesulphonic acid and α-aminoethanesulphonic acid. α-Aminosulphonic acids are generally quoted as being metabolic antagonists of the α-aminocarboxylic acids (Woolley 1947, 1952; Winzler, 1949). Initially, McIlwain (1941*a*) reported that the sulphonic acid analogues of glycine, alanine, valine and leucine inhibited the growth of a number of organisms. Subsequently, Spizizen (1943) found aminomethanesulphonic acid to inhibit the multiplication of a bacteriophage of *Escherichia coli*, and Ackermann (1952) found aminophenylmethanesulphonic acid and α-aminophenylethanesulphonic acid to be active inhibitors of the growth of influenza virus in tissue cultures. The fate of such compounds in the animal body would therefore be of particular interest from a chemotherapeutic standpoint. A complicating factor in studies with α-aminosulphonic acids, and one to which insufficient attention appears to have been paid, is the ease of their decomposition in solution. In the case of aminomethanesulphonic acid, Reinking, Dehnel & Labhardt (1905) and Salkowski (1918) reported that decomposition to sulphite occurred spontaneously in neutral aqueous solution, this being accelerated by heat and by the presence of acids and alkalis. McIlwain (1941*b*), however, stated that typical α-aminosulphonic acids, with the exception of the citronellal analogue, showed no appreciable decomposition to sulphite under anaerobic conditions at pH 7·6 and 37°, although breakdown was extensive at 100°.

The stability in solution of two compounds of this type, aminomethanesulphonic acid and α-aminoethanesulphonic acid, was reinvestigated. Solutions (0·02 M) of each compound in 0·1 M phosphate or veronal buffer were kept in stoppered flasks in a

Table 3. *Decomposition of aminomethanesulphonic acid and α-aminoethanesulphonic acid to sulphite and sulphate*

(Solutions (0·02 M) of each sulphonic acid in 0·1 M veronal buffer were kept 24 hr. at 37° before analysis.)

Compound	Conditions	Percentage of sulphonic acid converted		
		To sulphite	To sulphate	Total
Aminomethanesulphonic acid	Anaerobic, pH 7·6	14·8	21·1	35·9
α-Aminoethanesulphonic acid		7·0	25·0	32·0
Aminomethanesulphonic acid	Aerobic, pH 7·0	4·4	25·7	30·1
α-Aminoethanesulphonic acid		0	12·6	12·6
Aminomethanesulphonic acid	Aerobic, pH 8·0	8·0	32·9	40·9
α-Aminoethanesulphonic acid		0	74·1	74·1

thermostat at 37°. When anaerobic conditions were required, nitrogen was bubbled through the buffer during the preparation of the solutions and also throughout the duration of the experiment. Sulphite was determined iodometrically on samples withdrawn at intervals from the flasks. Sulphate was determined on solutions made up in veronal buffer only, as with phosphate buffer preliminary phosphate removal would have been necessary. Under the conditions described by McIlwain (1941*b*), α-aminoethanesulphonic acid was found to decompose slowly to sulphite (7 % breakdown after 24 hr.). Experiments using veronal buffer showed that conversion to sulphate, on the other hand, was high (25 % after 24 hr.). Over the same period aminomethanesulphonic acid was decomposed to a considerable extent to both sulphite and sulphate as shown in Table 3. In the case of either compound, the results obtained for sulphite formation in phosphate buffer and veronal buffer were identical. Further experiments, carried out under aerobic conditions at pH's 7·0 and 8·0, have been summarized in Table 3. The results obtained confirm the previous findings that the two sulphonic acids are unstable in solution. Under both aerobic and anaerobic conditions over the pH range 7–8 aminomethanesulphonic acid gave rise to as much as 9–12 % of sulphite in one hour. Under aerobic conditions this amount diminished during the next 24 hr. to the values given in Table 3, presumably as a result of air oxidation, whereas in nitrogen the amount of sulphite present increased to about 15 %. On account of their instability in aqueous solution at 37° these compounds were considered unsuitable for inclusion in the present series of experiments on rats. The experiments described above serve to emphasize that the mere absence of sulphite in a solution of an α-aminosulphonic acid does not justify the assumption that the compound is stable. The findings on growth inhibition of micro-organisms produced by α-aminosulphonic acids must therefore be accepted with reserve, and a careful study of the stability of individual compounds of this type is essential if studies involving them are to have any validity.

DISCUSSION

Salkowski (1873, 1876, 1918) reported that sodium isethionate, taurine and aminomethanesulphonic acid, when administered orally to the dog and rabbit, were metabolized to sulphate and thiosulphate, whereas sodium ethanesulphonate was excreted unchanged. Schmidt & Clark (1922) were unable to confirm the *in vivo* oxidation of the first two compounds in the dog. White, Lewis & White (1937) found that the rabbit partly oxidized orally administered taurine and L-cysteic acid, but not the injected compounds. Similar results for injected and ingested L-cysteic acid were obtained by Medes (1937) and Medes & Floyd (1942) in man. Among other published data on administered aliphatic sulphonic acids are the experiments of Flaschenträger, Bernhard, Löwenberg & Schläpfer (1934), in which sodium *n*-octanesulphonate, given by mouth to dogs, failed to increase the urinary sulphate output and Bronner & Schueller (1931) found sodium iodomethanesulphonate to be excreted mainly unchanged when administered intravenously to mice, rabbits and dogs. This was based on the absence of inorganic iodide in the urine during such experiments, and the recovery of 93 % of the administered organic iodine in the urine of dogs 24 hr. after the dose.

In the present series of experiments methanesulphonic acid, iodomethanesulphonic acid, isethionic acid, sulphoacetic acid, β-sulphopropionic acid, sulphamic acid and taurine, administered subcutaneously as their sodium salts, were found not to be oxidized to any significant extent to sulphate by the rat. This is in accord with the earlier findings by the workers referred to above, obtained with the rabbit, dog, mouse and man. Recorded instances of increases in sulphate excretion appear to be associated only with the oral administration of these compounds and it seems likely that the increases are the result of the action of intestinal flora (White *et al.* 1937).

Changes in the output of urinary neutral sulphur following the administration of the sodium salts of

methanesulphonic acid, iodomethanesulphonic acid and isethionic acid to rats showed that there was rapid and almost complete elimination of the sulphur of these compounds, indicating the lack of utilization of sulphur in this form. In the case of the first two compounds, it was found that this neutral sulphur increase was due largely, if not entirely, to the excretion of the unchanged acids, which were recovered from the urine as their phenylhydrazine salts. Flaschenträger *et al.* (1934) also recovered *n*-octanesulphonic acid unchanged from the urine of dosed dogs in the form of its barium salt. The behaviour of these sulphonic acids may be contrasted with that of taurine and L-cysteic acid, the sulphur of which is excreted only partly in the urine by the rat and other species.

There is so far no evidence of a direct pathway for the enzymic degradation of chemically stable aliphatic sulphonic acids to sulphate in the animal. Furthermore, with the exception of taurine and L-cysteic acid, these compounds do not appear to undergo any metabolic change *in vivo*.

SUMMARY

1. Six aliphatic sulphonic acids and sulphamic acid, administered to rats by subcutaneous injection as sodium salts in neutral solution, produced no significant increases in the excretion of urinary inorganic and ethereal sulphate.

2. The sulphur of injected methanesulphonic acid, iodomethanesulphonic acid and isethionic acid was excreted almost quantitatively in the neutral sulphur fraction of the urine. Injected methanesulphonic acid and iodomethanesulphonic acid were recovered in about 70% yield from the urine of dosed rats in the form of the phenylhydrazine salts.

3. Aminomethanesulphonic acid and α-aminoethanesulphonic acid undergo extensive decomposition to sulphite and sulphate in aqueous solution at 37° and are unsuitable for inclusion in biochemical experiments of the type described.

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The Oxidation of Dimethylthetin and Related Compounds to Sulphate in the Rat

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Sulphonium salts, such as the trimethylsulphonium and triethylsulphonium halides, possess certain of the pharmacological properties of quaternary ammonium compounds (Crum Brown & Fraser, 1872) and have been given some attention on this account in the past (Hunt & Renshaw, 1925, 1932; Ing & Wright, 1931). More recently, interest in sulphonium compounds has centred on the thetins and their role in transmethylation. Du Vigneaud (1942–3) first reported that dimethylthetin chloride (carboxymethyldimethylsulphonium chloride) $\{(CH_3)_2S^+CH_2CO_2H\}Cl^-$, could support the growth of rats fed on a methyl-deficient diet supplemented with homocystine, and that it could act, like choline and glycine betaine, as a methyl donor in the rat (Du Vigneaud, Moyer & Chandler, 1948). A related thetin, dimethyl-β-propiothetin, was also found to be an active methyl donor (Maw & Du Vigneaud, 1948*a*); this is of significance in view of its natural occurrence in a marine alga (Challenger & Simpson, 1947, 1948). Ethylmethylthetin is partly active in this respect, whereas diethylthetin, lacking the necessary methyl groups, is inactive (Maw & Du Vigneaud, 1948*c*). Dubnoff & Borsook (1948*a*), using rat, pig and guinea-pig liver suspensions, demonstrated the ability of dimethylthetin and dimethyl-β-propiothetin to methylate homocysteine anaerobically *in vitro* and showed that a specific transmethylase was involved (Dubnoff & Borsook, 1948*b*). Only one methyl group per molecule of thetin was transferred in these experiments. The methyl groups of these two thetins can also be rapidly oxidized to carbon dioxide by the rat (Ferger & Du Vigneaud, 1950).

A fairly clear picture has thus been established of the metabolic utilization of one of the methyl groups of dimethylthetin and related thetins, but information regarding the fate of the rest of the molecule, particularly of the sulphur, is lacking. The experiments described here are concerned with the extent to which the sulphur of such compounds is converted to sulphate by the intact rat, and with the nature of the possible intermediates in the path of sulphate formation. Dimethylthetin, in the form of its chloride, has been found to undergo rapid and extensive oxidation to sulphate. Experiments with seven other related sulphonium chlorides indicate that it is not a general catabolic reaction for this type of compound. In the limited series of compounds studied, there is evidence of a correlation between transmethylation and sulphate formation. It is considered that loss of a methyl group represents an initial and obligatory step in the catabolism to sulphate of dimethylthetin and probably other allied compounds.

MATERIALS AND METHODS

Preparation of compounds. The chlorides of dimethylthetin, ethylmethylthetin and diethylthetin were prepared by incubating a mixture of equimolar amounts of monochloroacetic acid and the appropriate sulphide at 35° for 24 hr., followed by washing of the sulphonium salt with ether and recrystallization from ethanol. Dimethyl-β-propiothetin chloride (β-carboxyethyldimethylsulphonium chloride) was most satisfactorily prepared by way of the bromide from methyl sulphide and β-bromopropionic acid (Challenger & Simpson, 1948). Sulphocholine chloride (β-hydroxyethyldimethylsulphonium chloride) and β-hydroxyethyl methyl sulphide were prepared as described by Maw & Du Vigneaud (1948*b*). Trimethylsulphonium, ethyldimethylsulphonium and triethylsulphonium chlorides were obtained from the iodides by treatment with AgCl. Sodium *S*-methylthioglycollate was made by treatment of the sodium derivative of methyl thioglycollate with methyl iodide. The resulting methylated ester was hydrolysed with dilute alkali, and after acidification, the *S*-methylthioglycollic acid was extracted with ether and converted to the Na salt. Sodium *S*-ethylthioglycollate was prepared in a similar manner from methyl thioglycollate and ethyl iodide. Sodium thioglycollate was prepared from a freshly distilled sample of the acid.

Plan of metabolism experiments. Pairs of male rats (wt. 120–160 g.) were used. The routine adopted in the animal experiments, the diet used, the urine collection and the determination of urinary sulphate were as in the preceding paper (Maw, 1953).

Administration of compounds. Although neutralized aqueous solutions of dimethylthetin chloride can be administered to rats orally or by injection in single doses of 15 mg. S/rat without any untoward effects, some of the other compounds, e.g. trimethylsulphonium and triethylsulphonium chlorides and sodium thioglycollate, are toxic at this level and caused death of the animals. All compounds were well tolerated, however, when administered by incorporation with the diet, and this mode of dosage was, therefore, adopted, since not only did it allow relatively

large doses to be employed, but the slow administration gave the maximum opportunity for absorbed compounds to be metabolized with the minimum of their renal excretion. The daily food consumption of each pair of rats was determined during an acclimatization period of 4–5 days. This amount of food was then supplied daily throughout the rest of the experiment. After a further control period of 4–5 days, the compound under study was mixed in with 1 day's allocation of food, and for a final period of 4–5 days the animals were fed on the diet alone. Where possible, the compounds were added to the diet in the dry state, but those sulphonium chlorides deliquescent were added in 4 ml. of water, the amount of compound present being calculated from the chloride content of the solution. The majority of compounds used did not cause any change in food intake when given with the diet. When, however, any food remained uneaten on the day of the administration, the uneaten portion was weighed and the actual amount of compound ingested then calculated. Urine was collected every 24 hr. from the beginning of the control period.

RESULTS

In an initial series of experiments a considerable proportion of an orally administered dose of dimethylthetin was found to be oxidized to sulphate by the rat, the resulting increase in urinary sulphate output being associated with the inorganic but not the ethereal fraction. This increase took place over a 2-day period, since the rats consumed the diet containing the compound mainly at night, towards the end of the 24 hr. period during which it was administered. It was therefore to be anticipated that catabolites of the thetin, such as sulphate, would be present in the urine on the second day after administration. The 24 hr. inorganic sulphate outputs then generally returned to pre-dosage levels, although in some experiments they were still significantly raised 4–5 days later. The excretion of inorganic sulphate during the control period was subject to as little variation as in previous experiments (Maw, 1953). Increases in sulphate output produced experimentally have therefore been assessed as the amounts excreted in excess of the average output for the control period. Table 1 gives the summarized results for four experiments and also includes results for three experiments in which neutralized solutions of dimethylthetin chloride were administered by injection. In these last experiments considerable conversion of the thetin to sulphate was again obtained, but the increase in urinary sulphate output was complete 24 hr. after the injection. The values for the percentage oxidation of injected doses were sufficiently close to those obtained when the compound was administered orally to indicate that the oxidation occurred in the tissues of the rat itself and was not to any great extent, if at all, the result of bacterial action in the intestine.

Table 1. *Oxidation of dimethylthetin chloride to sulphate by the rat*

Mode of administration	Dose (mg. S/rat)	Percentage of S of the dose excreted as inorganic sulphate: 1st day	2nd day	Total
By mouth (mixed with the diet)	16·0	57·9	0	57·9
	14·0	57·6	0	57·6
	16·1	61·1	7·6	68·7*
	8·0	47·1	9·6	56·7
By subcutaneous injection	15·3	53·2	3·9	57·1
	15·3	37·4	0	37·4
	15·3	43·7	0	43·7

* Inorganic sulphate excretion still significantly above the pre-dosage level 6 days after administration.

Table 2. *Oxidation to sulphate by the rat of compounds related structurally to dimethylthetin chloride*

(Compounds were administered by addition to the diet unless otherwise stated.)

Compound	Dose (mg. S/rat)	Percentage of S of the dose excreted as inorganic sulphate: 1st day	2nd day	Total
Diethylthetin chloride	13·2	0	0	0
	13·5	0	0	0
Ethylmethylthetin chloride	10·7	17·6	4·5	22·1
	14·6	22·1	4·3	26·4
Dimethyl-β-propiothetin chloride	13·0	24·9	42·0	66·9*
	13·3	38·9	27·4	66·3
Trimethylsulphonium chloride	6·8	0	3·8	3·8
	6·3	0	3·7	3·7
Ethyldimethylsulphonium chloride	6·0	0	0	0
	8·3	0	1	1
Triethylsulphonium chloride	10·1	0	0	0
Sulphocholine chloride	10·4	11·0	10·2	21·2
	13·6	28·2	12·4	40·6*
	13·6	21·4	0	21·4
	9·6	13·9	0	13·9
	14·1†	14·4	2·9	17·3
β-Hydroxyethyl methyl sulphide	15·4	48·6	12·6	61·2
	17·0	60·0	0	60·0

* Inorganic sulphate excretion still significantly higher than the pre-dosage level 4–5 days after administration.
† Administered by subcutaneous injection.

In view of the ease and extent of oxidation of dimethylthetin by the rat, the formation of sulphate from some related compounds was studied in order to see whether the process is specific for dimethylthetin or whether it is a general one for sulphonium compounds. The results obtained are shown in Table 2. When an increase in urinary sulphate was obtained, it took place over a 2-day period. Dimethyl-β-propiothetin was oxidized to sulphate

about as extensively as dimethylthetin itself; ethylmethylthetin was oxidized to a lesser extent, whereas diethylthetin did not form sulphate. Three simple alkylsulphonium salts, trimethylsulphonium, ethyldimethylsulphonium and triethylsulphonium chlorides were not significantly oxidized. Sulphocholine chloride, on the other hand, did form sulphate, but the percentage conversion ranged from 13·9 to 40·6. It is thus evident that oxidation to sulphate is not a general metabolic reaction for sulphonium salts.

Possible intermediates in sulphate formation. It is believed that in the animal dimethylthetin is converted to *S*-methylthioglycollic acid by transfer of a methyl group (Maw & Du Vigneaud, 1948*c*; Dubnoff & Borsook, 1948*b*), although this compound has not yet been isolated as a catabolite. It is also reasonable to suppose that the methyl donor ethylmethylthetin, on demethylation, gives rise to *S*-ethylthioglycollic acid. Sodium salts of these two alkylthioglycollic acids and thioglycollic acid itself were administered to rats; the percentages converted to sulphate are given in Table 3. Thioglycollic acid is known to be partly oxidized in this way in dogs and rabbits (Smith, 1893; Hill & Lewis, 1924). In the rat the percentages in two experiments were 35·5 and 38·8. The urine voided during the 2-day period after administration gave a negative test for SH groups but a marked positive reaction for SS groups (Brand, Harris & Biloon, 1930), suggesting partial conversion of the compound to dithiodiglycollic acid. *S*-Methylthioglycollic acid was oxidized to a considerable extent, but *S*-ethylthioglycollic acid less so. It is of interest that the values obtained for these two alkylthioglycollic acids correspond closely to those obtained for the thetins from which, it is suggested, they are derived. Table 2 includes results for the administration of β-hydroxyethylmethyl sulphide to rats. The ease with which this compound is oxidized to sulphate may implicate it as a metabolite of sulphocholine if the latter compound were to undergo enzymic demethylation.

Table 3. *Oxidation to sulphate by the rat of possible intermediates in the metabolism of thetins*

(Compounds were administered by addition to the diet.)

Compound	Dose (mg. S/rat)	Percentage of S of the dose excreted as inorganic sulphate: 1st day	2nd day	Total
Sodium thioglycollate	16·0	35·5	0	35·5
	15·1	37·3	1·5	38·8
Sodium *S*-methylthioglycollate	15·1	57·4	0	57·4
	11·8	56·4	8·0	64·4
Sodium *S*-ethylthioglycollate	15·2	25·5	4·0	29·5
	15·2	20·0	0	20·0

Many sulphonium compounds of the form $\{RS^{+}(CH_3)_2\}X^{-}$ may be degraded chemically in the presence of alkali to methyl sulphide, which is also evolved from the marine alga *Polysiphonia fastigiata* as a result of the enzymic breakdown of the naturally occurring dimethyl-β-propiothetin (Challenger & Simpson, 1947, 1948). Furthermore, the urines collected after the administration of sulphocholine to rats smelt of methyl sulphide, although whether its presence was the result of enzymic action, bacterial action, or simple chemical degradation in urine has not been studied. Methyl sulphide has therefore been examined as a likely intermediate in sulphate formation. It was administered as a solution in arachis oil either by injection or by incorporation in the diet. The results for its oral administration are undoubtedly subject to criticism on the grounds of its high volatility, and to offset losses from the food a larger amount of the sulphide was used (equivalent to 30 mg. sulphur/rat). When administered by injection it was clearly detectable in the breath of the rats within 1 hr. of dosing. In six experiments, no resultant increase in urinary sulphate was observed. Were methyl sulphide an intermediate in the catabolism of dimethylthetin to sulphate, some increase in sulphate output in these experiments might have been expected, considering the large proportion of dimethylthetin sulphur oxidized in this way.

DISCUSSION

Of the eight sulphonium chlorides administered orally to rats only dimethylthetin, dimethyl-β-propiothetin, ethylmethylthetin and sulphocholine caused an increase in urinary inorganic sulphate, indicating that the oxidation process involves a certain degree of enzymic specificity. The first three of these compounds are active as methyl donors in the rat (Maw & Du Vigneaud, 1948*a*, *c*), and the first two in liver suspensions (Dubnoff & Borsook, 1948*a*, *b*), in which one methyl group per molecule of thetin is transferred to homocysteine. The most likely intermediates in the path of oxidation of dimethylthetin and ethylmethylthetin to sulphate are their demethylated products, *S*-methylthioglycollic acid and *S*-ethylthioglycollic acid respectively. This is in accord with the finding that the two alkylthioglycollic acids undergo oxidation to sulphate in the rat. Diethylthetin is inactive in promoting rat growth on a homocystine-containing diet and is not lipotropic. It is not oxidized to sulphate, and this is most probably because, unlike ethylmethylthetin, no mechanism exists for its conversion to *S*-ethylthioglycollic acid. Trimethylsulphonium chloride is likewise not a methyl donor

(Du Vigneaud & Maw, unpublished observations) and is not oxidized to sulphate, possibly for a similar reason. It is considered that loss of a methyl group either by transmethylation or by other degradative reactions represents an initial and obligatory step in the catabolism to sulphate of dimethylthetin, ethylmethylthetin and probably dimethyl-β-propiothetin. The possibility that the two processes are quite separate metabolic pathways is not ruled out, but the case of diethylthetin argues against this.

Sulphocholine is oxidized to a certain extent and if a preliminary demethylation were involved it would give rise to β-hydroxyethyl methyl sulphide, which forms sulphate to a considerable extent. In growth studies with rats, sulphocholine supplied in a methyl-free diet has definite lipotropic activity but does not appear to act as an active methyl donor, judged by the growth criterion (Maw & Du Vigneaud, 1948*b*), although any definite conclusions are complicated by the toxic nature of the compound. However, Dubnoff (1949) has reported that sulphocholine can methylate homocysteine *in vitro* under aerobic, but not anaerobic, conditions, and he suggests that sulphocholine is initially oxidized to dimethylthetin, just as choline is oxidized to betaine before taking part in its transmethylation reactions. Under these circumstances sulphocholine would eventually be demethylated, but to *S*-methylthioglycollic acid rather than to β-hydroxyethyl methyl sulphide. The behaviour of sulphocholine *in vitro* needs further examination, but may not be irreconcilable with the rat-growth studies discussed, since *in vivo* the compound may be releasing methyl groups, either directly or after oxidation to the thetin, too slowly to be capable of acting as a sole methyl source. Dubnoff's findings would account for the oxidation of sulphocholine to sulphate, and would extend the relationship between transmethylation and sulphate formation in the sulphonium series.

The intermediates in the metabolism of *S*-methylthioglycollic acid and *S*-ethylthioglycollic acid to sulphate are not yet known. *S*-Methylthioglycollic acid is not a methyl donor either *in vivo* (Maw & Du Vigneaud, 1948*c*) or *in vitro* (Dubnoff & Borsook, 1948*b*). It might, however, give rise to thioglycollic acid or dithiodiglycollic acid by oxidative demethylation. The urines of rats fed dimethylthetin and *S*-methylthioglycollic acid invariably gave negative tests for SH and SS groups, although any thioglycollic acid formed endogenously might well have been metabolized as fast as it was produced. Other possible catabolites include methyl mercaptan, methanesulphonic acid and sulphoacetic acid. The last two compounds when administered to rats by injection are not significantly oxidized to sulphate (Maw, 1953).

Methyl sulphide also requires consideration as a possible catabolite of sulphonium compounds, since its non-enzymic formation from a number of aliphatic dimethylsulphonium salts takes place readily and since it is formed enzymically from dimethyl-β-propiothetin (Challenger & Simpson, 1947, 1948). Repeated administration of the sulphide to rats, orally or by injection, has never resulted in an increase in urinary sulphate output. No decisive conclusions as to its role as a precursor of sulphate can at present be drawn from these experiments, in which it was of exogenous and not endogenous origin. This is also true for the experiments with methanesulphonic and sulphoacetic acids. But it may be pointed out that if the oxidation of a sulphonium salt, such as dimethylthetin, to sulphate requires an initial demethylation; methyl sulphide could never be formed, except by an entirely separate catabolic process. It is hoped that *in vitro* experiments will provide further information concerning the reactions involved.

SUMMARY

1. Dimethylthetin chloride administered to pairs of rats was oxidized to inorganic sulphate to the extent of 57·6–68·7 % when given orally and 37·4–57·1 % when injected subcutaneously.

2. Of seven other sulphonium compounds only ethylmethylthetin, dimethyl-β-propiothetin and sulphocholine were oxidized in this way, indicating that the formation of sulphate is not a general mode of metabolism for this type of compound.

3. In the limited series of compounds studied, oxidation to sulphate was confined to sulphonium salts which are able to act as biological methyl donors, and it is suggested that demethylation is an initial and obligatory step in their catabolism to sulphate.

4. Possible intermediates in the oxidation are discussed.

It is a pleasure to thank Miss A. Hills for skilled technical assistance throughout this work.

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The Estimation of D- and L-Glutamic Acid in Proteins

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In connexion with studies on the glutamic acid content of tumour proteins it was necessary to estimate the respective amounts of the two optical isomers given on acid hydrolysis. The enzymic decarboxylation procedure of Gale (1945) was applicable to the L-isomer, but a search for a specific method for the D-isomer was not successful. Experience showed that it was not oxidized by the specific D-amino acid oxidase of kidney and that the activity of fungal oxidases (cf. Bender & Krebs, 1950; Emerson, Puziss & Knight, 1950) was very low. Moreover, the preparation from rabbit liver of an enzyme able to oxidize D-glutamic acid and D-aspartic acid (Still, Buell, Knox & Green, 1949; Still & Sperling, 1950) could not be repeated. Total glutamic acid was therefore estimated by an ion-exchange method (Consden, Gordon & Martin, 1948), which does not distinguish between the isomers, and the D-component found by difference. The present communication concerns the application of this two-step procedure to four purified proteins as well as to certain protein preparations and derivatives which have been reported by previous workers to contain a fair proportion of the D-isomer. In addition, opportunity is taken to present new evidence on the racemization of the L-isomer during acid hydrolysis.

EXPERIMENTAL

General methods

Nitrogen determinations were made by the micro-Kjeldahl procedure. Amino N was estimated by the method of Van Slyke (4 min. reaction time). L-Glutamic acid was estimated by the decarboxylase procedure of Krebs (1948), using a washed suspension of *Clostridium welchii* S.R. 12. Optical rotations were determined in a 4 dm. tube unless otherwise stated. Protein hydrolysates were prepared by boiling with 6N-HCl for 24 hr. Evaporations *in vacuo* were conducted below 40°.

Materials

The sample of L-glutamic acid hydrochloride used was recrystallized; $[\alpha]_D^{20} + 31{\cdot}4°$ (*c*, 4·0 in 2·5N-HCl). The other amino-acids were purified commercial samples. The following proteins were purified samples available in the laboratory; the N content is quoted on a moisture- and ash-free basis unless stated: Edestin, N = 18·65%; ox insulin, recrystallized according to Du Vigneaud, Miller & Bodden (1939), N = 15·58%; horse myoglobin, N = 16·6% (not corrected for ash). Preparations of casein and gelatin described below were made from commercial samples.

L-Pyrrolidonecarboxylic acid. L-Glutamic acid was heated as described by Wilson & Cannan (1937). The anhydride was extracted from aqueous solution with ethyl acetate, from which solvent it was twice recrystallized. $[\alpha]_D^{20} - 9{\cdot}92°$ (*c*, 7·5 in water).

Amino acid control mixtures. These were made up to simulate *A*, haemoglobin; *B*, gliadin; *C*, myoglobin (Table 1). *A* was rich in aspartic acid, *B* in glutamic acid and *C* had these two amino acids present in the proportions usually found in proteins.

Tobacco-mosaic-virus protein. This was prepared by heat coagulation of the nucleoprotein and was a gift from Dr J. D. Smith, Molteno Institute, Cambridge; N = 17·89% (not corrected for ash).

Dried cells of Lactobacillus casei. Strain 3253 of the National Collection of Type Cultures was grown for 50 hr. at 30° in 1 l. flasks of the medium described by Rodwell (1953). The flasks were chilled to 0° and the bacteria separated by centrifuging. They were washed thrice with

M-NaCl, once with 50% (v/v) aqueous ethanol, twice with 98% (v/v) ethanol, thrice with ether and then dried over P_2O_5 at 5 mm. Hg and room temperature. The weight of dried cells from 3 l. of culture was 2·78 g. (N = 10·03%).

Table 1. *Composition of amino-acid control mixtures*

Amino acid	Weight (mg.) A	B	C
DL-Alanine	295·7	23·8	44·2
DL-Valine	318·0	29·8	37·4
DL-Leucine	398·9	33·2	170·9
DL-Isoleucine	238·3	—	—
DL-Serine	230·3	62·2	22·7
DL-Threonine	174·0	—	59·7
L-Proline	156·2	150·6	23·0
DL-Tyrosine	207·6	34·3	22·5
DL-Phenylalanine	317·7	72·2	75·4
DL-Methionine	36·5	18·8	16·8
L-Cystine	40·8	29·7	—
L-Tryptophan	38·3	8·5	33·6
DL-Lysine hydrochloride	382·4	15·1	198·6
L-Histidine	—	21·0	108·7
L-Histidine dihydrochloride	464·5	—	—
L-Arginine hydrochloride	179·5	38·0	31·2
L-Aspartic acid	708·5	18·3	125·6
L-Glutamic acid hydrochloride	485·5	647·7	188·3
Ammonium chloride	121·7	294·1	—

Racemized products from casein. Dakin & Dudley (1913) reported that the glutamic acid of casein was completely racemized by dilute alkali at low temperature. 50·27 g. casein (air-dry), 500 ml. 0·5 N-NaOH and a few drops of toluene were placed in a 1 l. flask, the mixture shaken and then incubated at 37° for 23 days, shaking at intervals. The mixture was then filtered, the filtrate neutralized with H_2SO_4 and adjusted to 1 l. Glacial acetic acid was added to precipitate the 'racemized casein', which was then filtered, washed and dried. The collected filtrate and washings are referred to in Table 6 as 'racemized caseose'.

Racemized products from gelatin. 50 g. gelatin (air-dry) were treated as described by Dakin (1912). The incubation mixture was separated by dialysis in cellophan into an indiffusible 'protein' fraction and a diffusible 'peptide' fraction (Table 6).

Estimation of glutamic acid by means of Amberlite IR-4B

The procedure followed closely that of Consden *et al.* (1948): Amberlite IR-4B resin was stirred and washed by decantation with water or dilute HCl until the pH of the supernatant liquid reached and remained at the required value. A portion of the hydrolysate (10–20 mg. N) was placed on a 1 cm. diam. column containing 5 g. Amberlite IR-4B equilibrated to pH 3·4. The column was washed with 200 ml. water and the washings discarded. The dicarboxylic acids were eluted with 100 ml. N-HCl and the eluate taken to dryness repeatedly *in vacuo* to remove excess mineral acid. The residual hydrochlorides were dissolved in a minimum volume of water and transferred to a similar column equilibrated to pH 2·6. This column was eluted with 0·001 N-HCl, amino N being determined on successive 5 ml. fractions of the eluate. Under these conditions glutamic acid was eluted before, and distinctly separated from, the aspartic acid. The first column had to be prepared fresh for each determination, but the second, which maintained its pH indefinitely, could be used for several runs. After the first run the appearance of the glutamic acid and aspartic acid could be predicted in replicate analyses on the same column. The fractions containing glutamic acid were pooled, and amino N estimated on a sample. The purity of the glutamic acid was established by paper chromatography. Although no other amino acid was ever present as a contaminant in the fractions containing the dicarboxylic amino acids, there was a small quantity of aromatic amino acids eluted ahead of the glutamic acid. The eluate also contained traces of resin-depolymerization products which reacted with ninhydrin so that it was not possible to use the quantitative colorimetric method of Moore & Stein (1948) to estimate the glutamic acid. A small correction (2 μg. amino N/ml. eluate) was made for resin breakdown.

Racemization of L-glutamic acid under the conditions of protein hydrolysis

The extent of racemization was determined by polarimetry. To give maximum observed rotation a 4 dm. tube was used, which permitted a reading of about +5° to ±0·02° or ±0·4%. The results of a series of experiments are listed in Table 4: each recorded rotation represents the mean of twenty readings. In treatments (*b*) and (*c*) the glutamic acid hydrochloride was placed in a 500 ml. flask, 100 ml. 6 N-HCl were added and the solution taken to dryness *in vacuo* either once (*b*) or several times (*c*) as is customary when dealing with protein hydrolysates. The residue was redissolved in 2·5 N-HCl for polarimetry. In treatments (*d*) and (*e*) the hydrochloride was boiled under reflux for 24 hr. before taking to dryness as in (*b*) and (*c*) respectively.

Optical form of glutamic acid in control experiments

The recovery of glutamic acid on a column of Amberlite IR-4B was satisfactory (Table 2). Using the decarboxylase procedure the recovery of L-glutamic acid was not significantly different (Table 3), except when the control mixture was submitted to the usual conditions of acid hydrolysis, when it was consistently lower. This difference was recognized by Gale (1945), who applied a correction factor of +5% for racemization during hydrolysis (Tristram, 1948).

Table 2. *Recovery of glutamic acid from control mixtures on a column of Amberlite IR-4B*

Control mixture	Glutamic acid N (mg.) Taken	Found	Recovery (%)
A	1·155	1·154	99·9
A	1·155	1·156	100·1
A	1·155	1·184	102·6
B	3·954	3·939	99·6
B	3·954	3·961	100·2
		Mean	**100·5**
		S.E. ±	0·9

The results of different workers on the extent of racemization of glutamic acid under the conditions of protein hydrolysis are conflicting. Arnow & Opsahl (1940), Johnson (1940*b*), Jeannerat (1942), Klingmuller (1943) and Wieland & Paul (1944) observed a fall in optical rotation when pure

Table 3. *Recovery of* L-*glutamic acid from control mixtures by the enzymic method*

Mixture and treatment	No. of experiments	% Recovered (Mean ± S.E.)	% D-isomer by difference (Mean ± S.E.)
(*a*) L-Glutamic acid hydrochloride alone	10	99·7 ± 0·4	0·3 ± 0·4
(*b*) L-Glutamic acid hydrochloride + L-aspartic acid	4	99·3 ± 0·5	0·7 ± 0·8
(*c*) Control mixture *A**	4	97·8 ± 1·2	2·2 ± 1·0
(*d*) L-Glutamic acid hydrochloride refluxed 24 hr. in 6N-HCl and taken to dryness several times *in vacuo*	8	94·7 ± 0·5	5·3 ± 0·6
(*e*) Control mixture *A** treated as (*d*)	10	94·9 ± 0·7	5·1 ± 0·8
(*f*) Control mixture *C** treated as (*d*)	8	94·7 ± 0·7	5·3 ± 0·9

* Table 1.

Table 4. *Racemization of* L-*glutamic acid under the conditions of protein hydrolysis*

Treatment	No. of experiments	Mean $[\alpha]_D^{20}$ (2·5N-HCl, $c = 4$)	% D-isomer
(*a*) None	5	+31·43	—
(*b*) Solution concentrated to dryness once at 40°	2	+31·67	—
(*c*) Solution concentrated to dryness several times at 40°	3	+30·78	1·3
(*d*) Refluxed 24 hr. in 6N-HCl and taken to dryness once at 40°	2	+29·72	3·0
(*e*) As (*d*) but taken to dryness several times at 40°	6	+28·54	4·9

L-glutamic acid was heated with HCl. On the other hand, Kögl (1949) detected no change in rotation on treating L-glutamic acid with 10N-HCl for 24 hr. at either 115 or 130°.

Glutamic acid isolated from normal proteins after acid hydrolysis by Johnson (1940*a*), Chibnall, Rees & Williams (1943), Klingmuller (1943) and Wieland & Paul (1944) appeared to contain a small proportion of D-isomer as measured by the optical rotation. DL-Glutamic acid labelled with ^{15}N was added to a protein hydrolysate by Graff, Rittenberg & Foster (1940). From the isotope ratio in the L- and DL-glutamic acid isolated it was concluded that there was no D-isomer in the hydrolysate.

The present polarimetric results with L-glutamic acid (Table 4) confirm that racemization occurs during refluxing with 6N-HCl for 24 hr. and also when the usual technique is applied to remove the excess mineral acid. Under normal conditions applicable to protein hydrolysates (Table 4*e*) there is a decrease in the specific rotation which might result from destruction of 10% of the L-isomer or the inversion of 5% of it. The latter explanation would appear the more probable from the parallel loss found with the decarboxylase procedure (Table 3*d*, *e*, *f*). It is to be remembered, however, that the small loss recorded during the removal of excess mineral acid, observed with L-glutamic acid itself (Table 4*c*) might be due to anhydride formation (Wilson & Cannan, 1937), or perhaps to inversion during the closing or opening of the anhydride ring. A sample of L-pyrrolidonecarboxylic acid was accordingly passed through the usual hydrolysis procedure and, after removal of the excess mineral acid, 86% of the expected glutamic acid hydrochloride was crystallized out by saturating the aqueous solution with hydrogen chloride and allowing to stand at 0° for 3 days. The anhydride used had $[\alpha]_D^{20} - 9{\cdot}92°$; Foreman (1914) quotes −11·4°, and Abderhalden & Kautzsch (1910) −10·8° and −11·5° for the L-isomer and Ratner (1944) +11·4° for the D-isomer. Accepting the higher figure, the present preparation contained a maximum of 6% of the D-isomer. The isolated glutamic acid hydrochloride crystals ($[\alpha]_D^{20}$ +29·8° in 2·5N-HCl) contained about 3% of D-isomer and accounted for 86% of the pyrrolidonecarboxylic acid. If inversion does indeed occur during the reversible formation of the pyrrolidone ring, it is limited in extent, and would not make a large contribution to the total racemization observed under the conditions of protein hydrolysis.

RESULTS AND DISCUSSION

The experiments described above suggest that during the normal conditions of acid hydrolysis adopted in this laboratory about 5% inversion of L-glutamic acid occurs. On such a supposition the data given in Table 5 suggest that no significant amount of D-glutamic acid residues was present in the proteins concerned. Many replicate analyses were made in each case, and on statistical examination of the results it was found that individual estimates by the decarboxylase method were accurate to ±5% and by the Amberlite method to ±2%.

Protein preparations reported to contain large amounts of the D-isomer have been analysed in order to explore the application of the differential procedure to such material (Table 6). A significant proportion was found in the cells of *Lactobacillus casei*, confirming Camien, Salle & Dunn (1945), but not in tobacco-mosaic-virus protein, in contrast to the finding of Lewis & Olcott (1945), who used a different strain. All the products prepared by alkali treatment of casein contained, as was ex-

Table 5. *Isomers of glutamic acid in hydrolysates of purified proteins*

(Glutamic acid N as % total N.)

Protein	Both isomers Mean S.E.	L-Isomer Mean S.E.	D-Isomer (by diff.) Mean S.E.*	D-Isomer as % total glutamic acid
Edestin	9·83±0·2	9·60±0·2	0·23±0·27	2·4†
Horse myoglobin	9·33±0·2	8·71±0·3	0·62±0·22	6·6
Beef insulin	10·51±0·3	10·03±0·1	0·48±0·14	4·6

* S.E. of the difference between means in columns 1 and 2.
† Not significant.

Table 6. *Isomers of glutamic acid in hydrolysates of protein products reported to yield* D-*glutamic acid*

(Glutamic acid N as % total N.)

Product	Both isomers Mean	L-Isomer Mean S.E.	D-Isomer (by diff.)	D-Isomer as % total glutamic acid
Tobacco-mosaic-virus protein	7·38	7·05±0·22	0·33	4·5*
Cells of *Lactobacillus casei*	5·62	4·77±0·05	0·85	15·1
Alkali-treated proteins				
Casein	14·27	7·95±0·27	6·32	44·3
Caseose	11·90	6·57±0·11	5·33	44·8
Gelatin protein fraction	6·12	4·21†	1·91	31·2
Gelatin peptide fraction	5·29	3·38†	1·91	36·0

* Not significant.
† Insufficient estimates for statistical analysis.

pected from the work of Dakin & Dudley (1913), large amounts of the D-isomer. The results with gelatin are contrary to those reported by Dakin (1912) since here too the glutamic acid was largely racemized.

SUMMARY

1. The L- and total glutamic acid were estimated in three purified proteins and in protein preparations.

2. Five per cent of D-isomer was produced from L-glutamic acid under the conditions of acid hydrolysis and subsequent treatment of the hydrolysate for analysis.

3. D-Glutamic acid in excess of that estimated to be formed by inversion was found in hydrolysates of proteins which had been treated with dilute alkali, and in the cells of *Lactobacillus casei*, but not in four purified plant and animal proteins or in tobacco-mosaic-virus protein.

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The Enzymic Hydrolysis of Alkyl Fluoroacetates and Related Compounds

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Methyl fluoroacetate has been reported by Boyarsky and his co-workers (Boyarsky, Postel & Rosenblatt, 1948; Boyarsky, Rosenblatt, Postel & Gerard, 1949) to block conduction irreversibly in isolated frog nerves. We have found this to be a common property of alkyl fluoroacetates, bearing a certain relationship to their general toxicity in animals (Bergmann, Gitter & Shimoni, 1953). Fluoroacetic acid, which has no effect on nerve conduction, is also less toxic than its esters. This difference between the acid and its esters could be due to better penetration of the latter or could be related to the fact that esters in general possess a higher affinity to esterases than the corresponding acids (Bergmann, Wilson & Nachmansohn, 1950*a*). The latter possibility made it desirable to investigate the behaviour of esters of fluoroacetic acid towards acetylcholine esterase, although the observation of Doty & Gerard (1949) and Gerard and Doty (1950), that methyl fluoroacetate inhibits respiration of nerve cells earlier than their conduction, points to interference with the oxidative metabolism of the nerve as the basis of the fluoroacetate effect. The fluoroacetates proved to be very good substrates of specific and unspecific esterases, in accordance with the observations of Adams & Whittaker (1950) on chloro- and bromoacetates, and revealed some new aspects of esteratic activity, which are dealt with in this and subsequent papers.

MATERIALS AND METHODS

Electric organ choline esterase. This was prepared from *Torpedo marmorata* (obtained through the generosity of Prof. Bacchi, Marine Biological Station, Naples, Italy) or *Electrophorus electricus*. The purified preparation diluted 3000 times produced 8 μmoles CO_2/ml./hr., with acetylcholine (ACh), $3{\cdot}3 \times 10^{-3}$ M, as substrate.

Liver esterase. This was prepared from dog's liver, as described earlier (Bergmann & Shimoni, 1952). The enzyme concentrate, diluted 100 times, produced 6·6 μmoles CO_2/ml./hr. with 0·43 M-diacetin as substrate.

Enzymic activity at 23° was measured by the Warburg manometric method, using gelatin-bicarbonate buffer in an atmosphere of 95% air and 5% CO_2. Since several substrates required the addition of ethanol for complete solution, the influence of ethanol on these enzymes was studied (Fig. 1). It is evident that the choline esterase is inhibited much more by a given percentage of ethanol in the medium than is liver esterase. When ethanol was present, enzymic activity was calculated by multiplying the experimental rate of hydrolysis (in μmoles CO_2/ml./hr.) by the factor 100/(100 – % ethanol inhibition). In the experiments to be described no concentrations higher than 1·5% (v/v) ethanol for choline esterase and 3% for liver esterase were used.

Substrates. The alkyl fluoroacetates were a gift of Blank & Bergmann (1952). Some of the relevant physical properties of these esters, many of which are new compounds, are summarized in Table 1. The densities were determined approximately by weighing on an analytical balance a sample, the volume of which had been measured with a pipette; this was done in order to avoid much handling of these volatile and extremely toxic substances. The substrates were dissolved first in the quantities of ethanol indicated in Table 1 whenever necessary, and then brought with buffer to a standard concentration; from this stock solution dilutions were made as required. The rate of enzymic hydrolysis was determined by allowing for spontaneous hydrolysis measured at each substrate concentration with a control solution devoid of enzyme, but of otherwise identical composition. Ethyl lactate was a commercial preparation (Stoney-Mueller Inc., N.J.). Ethyl glycollate and ethyl methoxyacetate were prepared according to Fischer & Helferich (1911), and methyl nitroacetate according to Bouveault & Wahl (1904). All other esters were Eastman-Kodak products and were purified by fractional distillation. Diacetin was a gift of British Industrial Solvents Ltd. and Mecholyl of Merck, Rahway, N.J. We synthesized ethyl trifluoroacetate according to Gilman & Jones (1943), but the action of esterases on this substrate could not be measured, since it undergoes very rapid hydrolysis in water.

RESULTS

Acetylcholine esterase

p*S*-activity curves for the hydrolysis of *n*-alkyl fluoroacetates by acetylcholine esterase are given in Fig. 2. As far as their solubility permitted us to cover a sufficient range of concentrations, they all show maxima, which become higher with increasing chain length, thus producing a steeper slope of the ascending part of the curves. At the same time the p*S* optimum shifts to higher values. For comparison of the relative affinity of the members of this series two properties are thus available: (*a*) the position of the p*S* maximum, (*b*) the height of this maximum.

Table 1. *Physical properties of alkyl fluoroacetates and related esters*

Compound	B.p. (°) at 760 mm.	Density at 26°	Maximum concn. in buffer (M)	% Ethanol added	Spontaneous hydrolysis
		(1) Fluoroacetates			
Methyl	103	1·15	$3·3 \times 10^{-1}$	None	Fast
Ethyl	119	1·08	$4·2 \times 10^{-1}$	2	Fast
n-Propyl	137	1·00	$1·6 \times 10^{-1}$	3	Appreciable
n-Butyl*	154	1·00	$3·1 \times 10^{-2}$	2	Negligible
n-Amyl*	171	0·96	$1·3 \times 10^{-2}$	3	
n-Hexyl*	189	0·96	$3·1 \times 10^{-3}$	None	
*iso*Propyl*	124	1·01	$2·2 \times 10^{-2}$	2	
*iso*Butyl*	150	1·01	5×10^{-2}	3	
tert.-Butyl*	133–135	0·98	$3·1 \times 10^{-2}$	None	
		(2) Other halogenoacetates			
(*a*) Chloroacetates					
Methyl	131·5	1·18	$3·1 \times 10^{-1}$	None	Very fast
Ethyl	144·2	1·16	$9·4 \times 10^{-2}$	2	Slow
(*b*) Bromoacetates					
Methyl	147	1·62	$2·1 \times 10^{-1}$	None	Very fast
Ethyl	159	1·51	$7·8 \times 10^{-2}$	None	Very fast
		(3) Acetates			
Methyl			2·5	None	Negligible
Ethyl			$7·8 \times 10^{-1}$	None	
n-Propyl			$1·6 \times 10^{-1}$	None	
n-Butyl			$4·2 \times 10^{-2}$	1	
n-Amyl			$1·2 \times 10^{-2}$	None	
		(4) Miscellaneous esters			
Ethyl glycollate	160	1·08	$3·1 \times 10^{-1}$	None	Slow
Ethyl lactate	154	1·03	$3·1 \times 10^{-1}$	None	
Ethyl methoxyacetate	140–141	0·97	$3·1 \times 10^{-1}$	None	
Methyl nitroacetate	82 (18 mm.)	1·21	$3·1 \times 10^{-1}$	None	

* New compounds.

In Figs. 3 and 4 both these properties are plotted as functions of N, the number of carbon atoms in the alkyl chain. Instead of the rate of hydrolysis at the pS optimum the rate at any fixed pS value can be used for comparison, provided this value is on the same side of the maximum for all substrates and the curves do not intersect. If these conditions are fulfilled, such a comparison will give at least the correct order of activities (see Fig. 4, curve 2).

Fig. 2 also demonstrates that ACh is hydrolysed about 60% faster than *n*-amyl fluoroacetate. We confirmed for one substrate, namely, *n*-butyl fluoroacetate, the linear relationship between rate of hydrolysis and enzyme concentration. This enabled us to compare activities, even when different enzyme concentrations had to be used for different substrates.

The sequence established in Figs. 3 and 4 is in contrast to the results of Mounter & Whittaker (1950) on *n*-alkyl acetates, reproduced in Fig. 4, curve 3, which show a maximum at $N = 4$. This difference may be due to the use of enzyme from a different source and of a different degree of purity by these authors. However, since so far erythrocyte choline esterase has shown identical properties with the enzyme from electric organ (Augustinsson, 1949), we are inclined to ascribe the difference in results to the method used. Mounter & Whittaker calculate the final 'effective' concentration of their substrates as 0·1 M, assuming that all of the added ester is in solution, which is by no means always the case (compare Table 1, 4). We have redetermined with our enzyme the pS-activity curves for alkyl acetates, using 'true' solutions. The limited solubility of these esters and their precipitating effect on high concentrations of the enzyme permitted only partial determination of these curves (Fig. 5). From our data it is clear that frequent crossing takes place and therefore comparison of activities is not too reliable. However, as far as can be judged from Fig. 5, at p$S = 1$ the order of activity will be identical with that of the fluoroacetate series and in any case will not show a maximum at $N = 4$, as claimed by Mounter & Whittaker (1950).

Branching has a profound influence on the speed of hydrolysis as shown in Fig. 6. Although the total number of carbon atoms increases, the maximal rate decreases in the order ethyl : *iso*propyl : *tert.*-butyl = 19 : 3·5 : 1. *iso*Butyl is split about half as rapidly as *n*-butyl. The decrease is less pronounced

in the series ACh : Mecholyl = 2 : 1. It is noteworthy that the latter compound exhibits a well-pronounced pS optimum, in contrast to the findings of Mendel, Mundell & Rudney (1943) on erythrocyte enzyme.

The chloro- and bromo-acetates behaved similarly to the fluoroacetates; especially the bromo derivatives which exhibited well-pronounced maxima (Fig. 7). However, the order of activity of the methyl and ethyl esters is reversed for bromoacetate. It may be mentioned that the optimal pS values for the three ethyl halogenoacetates are in the order F < Br < Cl, and the maximum rate decreases in the order F > Br > Cl.

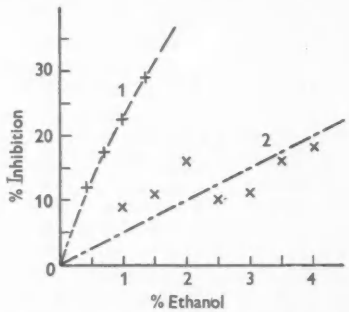

Fig. 1. Inhibition of esterases by ethanol. 1, Hydrolysis of ethyl fluoroacetate, 1×10^{-1} M, by acetylcholine esterase, 1:600; 2, hydrolysis of n-propyl fluoroacetate, $2{\cdot}7 \times 10^{-2}$ M, by liver esterase, 1:150.

Thus higher rate of hydrolysis is associated with a lower pS maximum. The methyl esters, however, follow a different order. Ethyl iodoacetate could not be measured very accurately, due to its rapid spontaneous hydrolysis, but the values obtained indicate a rather low degree of activity.

Inhibitory effects. Other electronegative substituents in the acetyl radical all produce a negligible hydrolytic rate (OH, OCH_3 and NO_2). This, however, is no proof of low affinity for the enzyme since these esters inhibit the hydrolysis of ACh. The same is true for *tert.*-butyl fluoroacetate, which is not split at all at an enzyme dilution of 1 : 600. Their I_{50} values are given in Table 2, including also butyrylcholine. This shows that an ester may form a rather stable complex with the enzyme, although this complex is not split at an appreciable rate. In accordance with previous observations (Bergmann & Shimoni, 1952), quarternary ammonium salts inhibit the hydrolysis of fluoroacetates.

In order to shed more light on the factors determining the rate of enzymic hydrolysis, a corresponding series of experiments was carried out with an unspecific esterase.

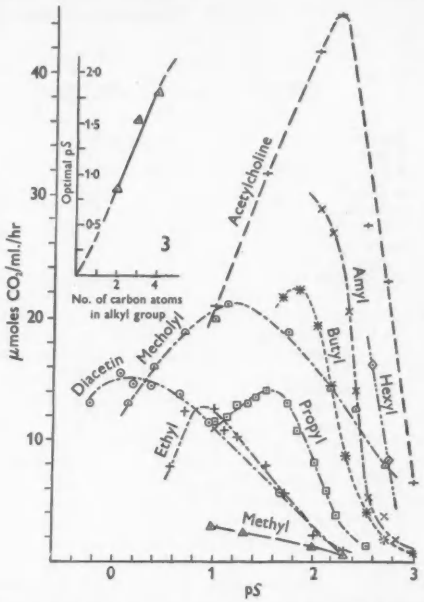

Fig. 2. Hydrolysis of n-alkyl fluoroacetates by acetylcholine esterase, 1:600. Mecholyl and diacetin were measured at the same enzyme concentration. For ACh an enzyme dilution of 1:1800 was used, and the rates then multiplied by 3 for comparison with the other esters.

Inset. Fig. 3. Optimal pS values of n-alkyl fluoroacetates as function of number of carbon atoms in the alkyl chain.

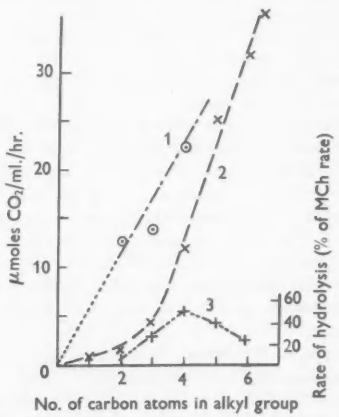

Fig. 4. Rate of hydrolysis of n-alkyl fluoroacetates as function of number of carbon atoms in the alkyl chain. 1, Rates at optimal substrate concentrations; 2, rates at pS 2·2; 3, relative rates of hydrolysis of n-alkyl acetates, according to Mounter & Whittaker (1950).

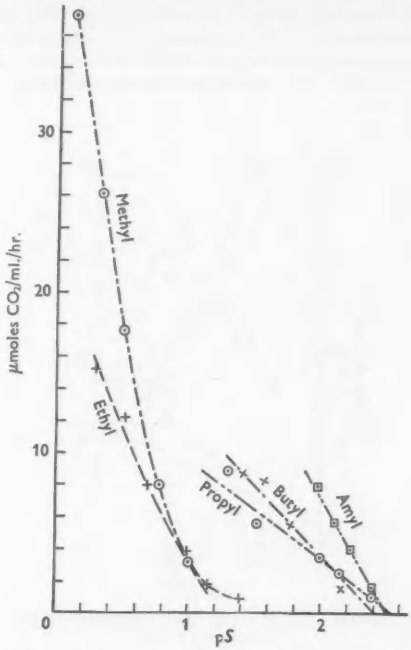

Fig. 5. Hydrolysis of *n*-alkyl acetates by acetylcholine esterase, 1 : 180.

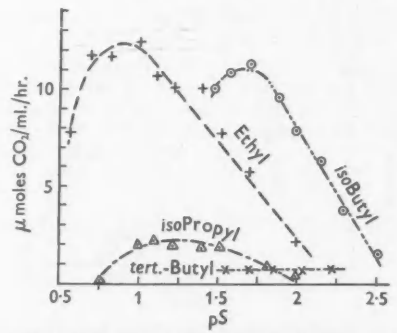

Fig. 6. Rate of hydrolysis of branched alkyl fluoroacetates by acetylcholine esterase, 1 : 600. The *tert.*-butyl ester was tested at an enzyme dilution 1 : 33 and the experimental values divided by 18 for comparison.

Liver esterase

The p*S* activity curves for the hydrolysis of fluoroacetates by liver esterase, as shown in Fig. 8, again present well-shaped maxima. It is very remarkable that whereas ACh possesses the highest maximum on choline esterase, it shows towards liver esterase an activity inferior even to diacetin so that a maximum can no longer be observed. We see in this fact a clear indication that two different principles are involved in the formation of ES_2- (or ES_n-) compounds in the case of fluoroacetates

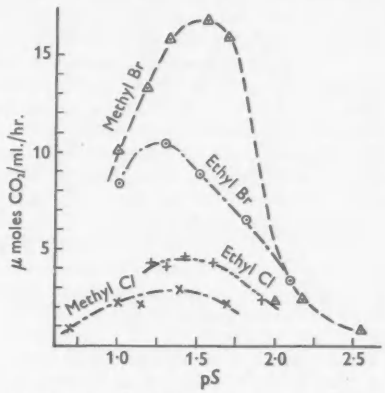

Fig. 7. Hydrolysis of chloro- and bromo-acetates by acetylcholine esterase, 1 : 600.

Table 2. *Inhibitory effect of various esters on acetylcholine esterase*

(The enzyme was incubated with the inhibitor for 1 hr. at 27°, then the substrate was tipped in and activity determined manometrically. For inhibitors 1–6, ACh, $3{\cdot}3 \times 10^{-3}$ M, was the substrate and final enzyme dilution was 1 : 3000. For inhibitor 7, *n*-propyl fluoroacetate, $1{\cdot}2 \times 10^{-2}$ M, was used as substrate and the enzyme was diluted 1 : 200. The concentration of inhibitor, required for 50% inhibition, was evaluated graphically.)

Inhibitor	I_{50} (M)
(1) Ethyl methoxyacetate	2×10^{-2}
(2) Ethyl glycollate	$3{\cdot}5 \times 10^{-1}$
(3) Ethyl lactate	2×10^{-1}
(4) Methyl nitroacetate	$2{\cdot}5 \times 10^{-2}$
(5) *tert.*-Butyl fluoroacetate	1×10^{-2}
(6) Butyrylcholine	$2{\cdot}5 \times 10^{-3}$
(7) Tetraethylammonium bromide	7×10^{-4}

on the one hand, and ACh on the other (see Discussion). Although it is impossible to demonstrate the presence of p*S* optima for the higher alkyl fluoroacetates due to limited solubility, it is apparent that the p*S* maxima are much more crowded and therefore the individual curves intersect frequently. Any comparison of rates becomes therefore arbitrary. From the limited data available it may be guessed that activity alternates between even and odd numbers of carbon atoms, since ethyl > methyl, *n*-butyl > *n*-propyl, *n*-hexyl > *n*-amyl and ethyl > *n*-propyl, *n*-butyl > *n*-amyl. However, for *n*-alkyl acetates no such regularity can be deduced from the curves in Fig. 9. But it can be stated that butyl appears to possess the smallest hydrolytic rate in

the homologous series. Ethyl chloro- and bromo-acetates show again pS maxima (Fig. 10), but the ethyl halogenoacetates exhibit now the following order of optimal pS values: Br > Cl > F, and the order for the maximum rates is Br > Cl > F, although the absolute differences are very small. Again, ethyl iodoacetate shows a low rate of hydrolysis.

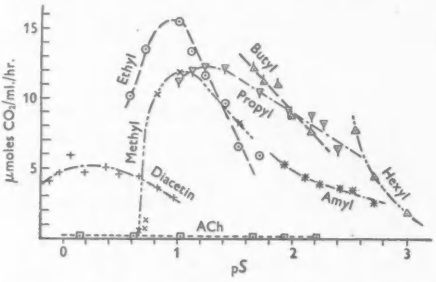

Fig. 8. Hydrolysis of *n*-alkyl fluoroacetates by dog's liver esterase, 1 : 150.

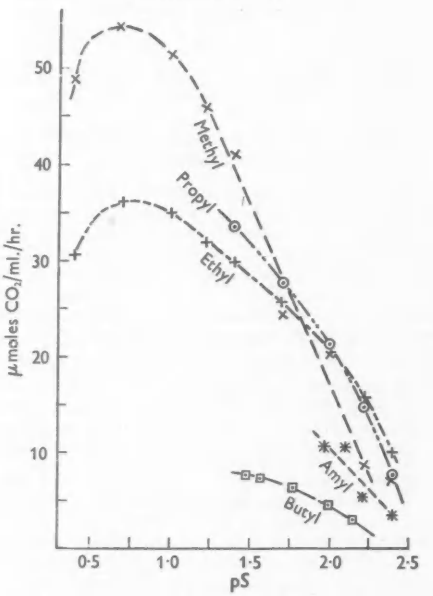

Fig. 9. Hydrolysis of *n*-alkyl acetates by liver esterase, 1 : 25.

The effect of branching is demonstrated by the curves in Fig. 11. Activity decreases again in the order ethyl : *iso*propyl : *tert.*-butyl = 15 : 13 : 1, whereas *iso*butyl approximately reaches the rate of the *n*-butyl derivative. Mecholyl is hydrolysed at a very low rate (about ¼ of ACh). Substituents other than halogen give considerable activity and eventually show a pS maximum (Fig. 12). Methyl nitroacetate could be measured only at an enzyme dilution of 1 : 10, which indicates low affinity for

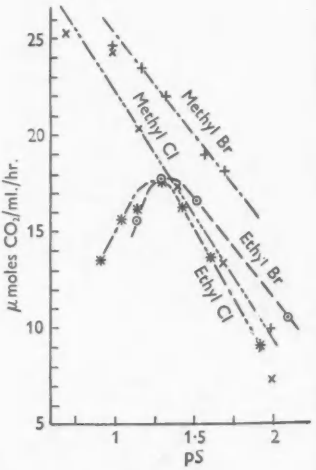

Fig. 10. Hydrolysis of chloro- and bromo-acetates by liver esterase, 1 : 150.

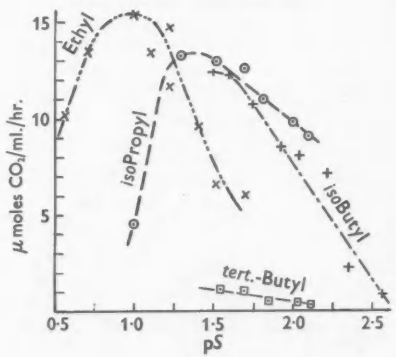

Fig. 11. Hydrolysis of branched alkyl fluoroacetates by liver esterase, 1 : 150. *tert.*-Butyl was measured at an enzyme dilution 1 : 10 and the experimental values divided by 15 for comparison.

the methyl ester. Unfortunately, the ethyl ester was not available.

We have also investigated the inhibitory effect of *tert.*-butyl fluoroacetate on the hydrolysis of *n*-propyl fluoroacetate by liver esterase and found no inhibition up to a concentration of the tertiary ester of 2×10^{-2}M. The same is true for the system liver esterase-diacetin, where Mecholyl at 7×10^{-1}M does

not cause inhibition. For the liver enzyme, in contrast to choline esterase, negligible reaction rate appears to indicate that the *ES*-complex is not formed to an appreciable degree.

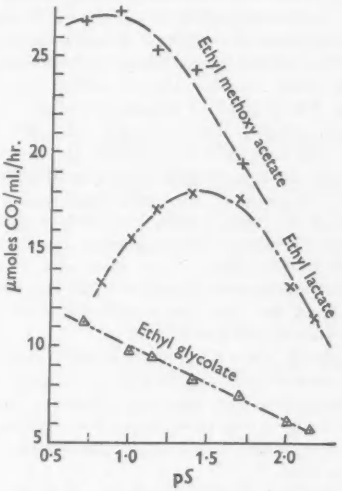

Fig. 12. Hydrolysis of miscellaneous esters by liver esterase, 1 : 150.

DISCUSSION

In a previous paper (Bergmann, Wilson & Nachmansohn, 1950*b*) we ascribed the occurrence of a Murray-Haldane curve for the system ACh-choline esterase to the presence of at least two negative groups in the neighbourhood of the esteratic site, leading to a sufficiently small value of K_2, the dissociation constant of Eqn. (2).

$$E+S \underset{k_2}{\overset{k_1}{\rightleftarrows}} ES; \quad ES \xrightarrow{k_3} ES^* \rightarrow E+P; \quad K_m=\frac{k_2+k_3}{k_1}, \quad (1)$$

$$ES+S \underset{k_5}{\overset{k_4}{\rightleftarrows}} ES_2; \quad K_2=\frac{k_5}{k_4}. \quad (2)$$

Since coulombic attraction cannot play a role in the combination halogenoacetate-choline esterase, the pronounced auto-inhibition of these esters must be ascribed to the presence of the halogen atom, which increases the electrophilic character of the carbonyl carbon. We therefore conclude that a bell-shaped p*S*-activity curve appears whenever substrates exhibit a high degree of affinity, affinity being expressed, e.g., by the magnitude of both K_m and K_2. It would appear at first glance that this statement invalidates the above conclusion on the combination of ACh with the enzyme surface. However, the experiments with liver esterase give a clear-cut answer: the halogenoacetates still exhibit a p*S* maximum, but not ACh. Therefore we conclude that two different principles are involved in the auto-inhibition of hydrolysis by acetylcholine esterase. In ACh the low affinity of acetates, as exemplified by diacetin or ethyl acetate, is overcome by the electrostatic attraction to the anionic sites. In halogenoacetates affinity must be mainly a function of the electronegativity of the substituent. Since ion-ion attraction is much stronger than ion-dipole or dipole-dipole interaction, we have a reasonable explanation for the outstanding affinity of ACh towards choline esterase. Since coulombic forces are also available for the combination with Mecholyl or butyrylcholine, this explains why Mecholyl is split half as fast as ACh, whereas *iso*-propyl fluoroacetate is hydrolysed at only one-fifth the rate of the ethyl ester. Similarly, the high affinity of butyrylcholine becomes evident from its I_{50} value (Table 2). Therefore the small rate of hydrolysis of butyrylcholine is due to the low rate of formation of an activated complex ES^* (small value of k_3, Eqn. (1)) from *ES*.

The auto-inhibition of neutral esters is difficult to reconcile with the original assumption of Haldane that two points of attachment are required in these cases for the combination enzyme-substrate. Since we assume that G_1, the nucleophilic group of the enzyme (Wilson & Bergmann, 1950*a*), combines with the carbonyl carbon and G_2, the electrophilic group, with the ether or carbonyl oxygen, the distance between G_1 and G_2 must be commensurable with the bond length of C—O or C=O (1·2–1·5 Å). Therefore, there is not sufficient space available to allow the combination of G_1 with one ester molecule and of G_2 with another one, since the van der Waals radii of atoms are at least twice as big as their bond radii.

Regarding the influence of halogen atoms, the following consideration will show that the halogenoacetates follow a simple rule on liver esterase. According to the procedure of Hammett (1940) a straight-line relationship should be expected between the dissociation constants of the halogenoacetic acids and the equilibrium constants of the enzymic hydrolysis. We have selected for comparison the Michaelis-Menten constants, which were determined approximately from a plot of s/v against S, neglecting the dissociation constants of the esteratic site (Wilson & Bergmann, 1950*b*). Although the K_m values are therefore not very accurate, they show for liver esterase linear proportionality as required by Eqn. (3) (Fig. 13, curve 2):

$$pK_m=A.pK_a+B. \quad (3)$$

The linear relationship is remarkable since the Hammett equation usually does not apply to aliphatic compounds. We may conclude that in the

systems under consideration proximity effects do not interfere seriously and the electron displacement due to the influence of the α-substituent is the decisive factor. Therefore the K_m values of the system halogenoacetate-liver esterase follow the order of electronegativity of the halogens (F = 4·0; Cl = 3·0; Br = 2·8; cf. Pauling, 1948). It should be mentioned that this result is in contrast to the conclusions reached by Adams & Whittaker (1950) for plasma choline esterase, for which van der Waals forces appear to play a decisive role.

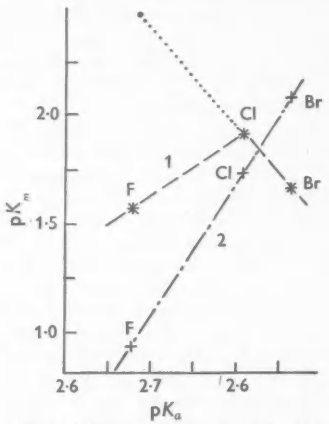

Fig. 13. Michaelis-Menten constants as function of acid dissociation constants of halogenoacetic acids. ($pK_m = A.pK_a + B$.) 1, for choline esterase; 2, for liver esterase.

Entirely different results were obtained with acetylcholine esterase. Curve 1 in Fig. 13 shows a maximum for chlorine. Since the negative sites introduce in this system ion-dipole interaction as a new force between enzyme and substrate, Adams & Whittaker (1950) calculated an affinity ratio for

$$\frac{\log K_{Cl}}{\log K_{Br}} = \frac{0{\cdot}40}{0{\cdot}55} = 0{\cdot}74.$$

However, their experimental figures, determined by the 'competition' method, did not confirm their calculation.* Since our K_m values are $1{\cdot}3 \times 10^{-2}$ for chloroacetate and $2{\cdot}2 \times 10^{-2}$ for bromoacetate, we find for the above ratio the value 0·87. Accepting this as a satisfactory agreement, we should expect fluoroacetate to possess a much smaller K_m, since the polarizability of a substituent is related to its molecular refraction. Actually the value is $2{\cdot}8 \times 10^{-2}$, i.e. greater than for the other halogenoacetates. Therefore fluorine does not occupy the position indicated by the dashed line in Fig. 13. A possible explanation for this abnormality will be given in a subsequent paper.

The description of the enzymic hydrolysis as a nucleophilic attack of the group G_1 in the active surface on the carbonyl carbon (Wilson & Bergmann, 1950*a*) explains the effect of changes in the alkyl chain to a certain degree. Since apparently there is enough space on the active surface of choline esterase for long alkyl chains, we should expect increased affinity due to larger van der Waals forces. This fact and, in addition, the decrease of activation energy, observed in the hydrolysis of homologous esters on cation-exchange resins (Davies & Thomas, 1952), both will explain the increase of rates with increased chain length. Apparently the effect of the dispersion forces on liver esterase is much smaller, since the observed p*S* maxima for the homologous series of fluoroacetates are crowded together.

Branching of the alkyl chain should decrease the rate of second-order hydrolysis (Hammett, 1940), as is observed for both enzymes. However, the effect of branching is less pronounced for liver esterase, suggesting again that steric hindrance is less effective here.

Our results contribute to a better definition of the specificity of acetylcholine esterase, for which a combination of factors is responsible: limited space for the acyl 'tail' of the substrate, the size of which determines the rate of conversion of *ES* into *ES**; large space for the alkyl group of the ester, as long as its side chains do not produce steric interference; the presence of negative charges in the neighbourhood of the esteratic site. All these factors are possibly various expressions of the influence of the large protein molecule on the active surface, which in 'unspecific' esterases is supposed to have a similar chemical constitution, but to be more free of environmental influences. Further studies, especially on serum choline esterase, will serve to define more sharply the differences between the various kinds of esterases and thus their specificity.

SUMMARY

1. Esters of halogenoacetic acids usually possess a bell-shaped p*S*-activity curve when hydrolysed by specific or unspecific esterases.

2. In the homologous series of *n*-alkyl fluoroacetates, when hydrolysis is effected by acetylcholine esterase, the p*S* optimum shifts to higher p*S* values with increasing chain length. Simultaneously the maximum rate of hydrolysis increases.

3. Fluoroacetates with a branched alkyl chain give a much lower rate on both types of esterases, the effect being greater on choline esterase than on liver esterase. This fact supports the assumption of

* It should be mentioned that this method is applicable only if the p*S*-activity curve is S-shaped. Our results show that no 'limiting' velocities exist for halogenoacetates.

nucleophilic attack of the group G_1 in the esteratic site on the carbonyl carbon of the ester.

4. Esters with a negligibly low rate of hydrolysis may prove to be efficient, reversible inhibitors of acetylcholine esterase. However, no such case has been observed on liver esterase.

5. The pK_m values (Michaelis-Menten constants) of the system ethyl halogenoacetates-liver esterase can be represented as a linear function of pK_a (acid dissociation constants), thus obeying the Hammett equation

$$pK_m = A.pK_a + B.$$

The corresponding curve for choline esterase, however, possesses a maximum for chloroacetate. It is concluded that fluoroacetate behaves abnormally towards the latter enzyme.

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A Dynamic Osmometer for Accurate Measurements on Small Quantities of Material: Osmotic Pressures of Isoelectric β-Lactoglobulin Solutions

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The osmometer described in the first part of this paper can be used for accurate measurements on quantities of solution of the order of 0·5 ml. The results obtained with it on unbuffered solutions are presented in the second part; they are of considerably greater accuracy than any comparable measurements known to the author, except for one series of measurements which required about ten times as much solution.

Part I. Design and use of the osmometer

PRINCIPLE AND THEORY

A rigid semi-permeable sac is placed in one limb of a U-tube with protein solution on one side and diffusate material on the other. Flow across the membrane is measured by observing the movement of a droplet in a narrow section at the bottom of the U-tube and is controlled by means of a manometer which enables pressures, greater or less than the osmotic pressure, to be applied to one limb of the U-tube. The pressure corresponding to zero flow is the required osmotic pressure subject to corrections for the following effects: (*a*) any difference in levels of the solutions in the two U-tube limbs; (*b*) differences in densities of the protein solution and diffusate—a very small effect; (*c*) differences between the surface tension rises in the two limbs.

Fig. 1 illustrates the hydrostatics of the system in more detail. Let the pressures and densities be as shown, and the membrane rigid and semi-permeable.

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At equilibrium:

Δ O.P., the osmotic pressure difference between solutions A and B
$= \{P_B + h_B\rho_B\} - \{P_A + h_A\rho_A\}$
$-$ the difference in upward pressure due to surface tension effects in A and B,

or, if $\rho_B - \rho_A = \Delta\rho$,

Δ O.P. $= \{P_B - P_A\} + (h_B - h_A)\,\rho_A + h_B\,\Delta\rho - \{$the surface tension effects$\}$.

The applied pressure at zero flow is $\{P_B - P_A\}$; and $(h_B - h_A)\,\rho_A$ is correction (a) which can be measured directly in the osmometer, since ρ_A is known. The last two terms are corrections (b) and (c), and are obtained by using a different U-tube in which the rigid semi-permeable membrane is

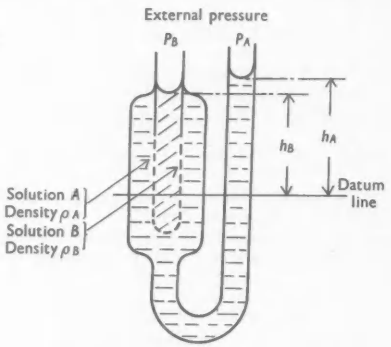

Fig. 1. Diagram to illustrate theory of corrections to a reading of osmotic pressure.

replaced by a completely flexible but impermeable sac in a semi-collapsed state. This new system is allowed to come to equilibrium with both of the U-tube limbs open to the atmosphere. Using dashes to represent the equilibrium heights under these conditions, it is clear that, provided the flexible sac does not exert any pressure,

$(h'_B - h'_A)\,\rho_A + h'_B\,\Delta\rho$
$-$ the surface tension effects = zero.

If the dimensions of the surface tension U-tube are the same as those in the osmometer, so that the surface tension rises are the same and $h_B \simeq h'_B$, then

$$\underbrace{(h'_B - h'_A)\,\rho_A}_{\text{in the surface tension U-tube,}} = \underbrace{\text{the surface tension effects} - h_B\,\Delta\rho}_{\text{in the osmometer}}$$

Hence

$$\Delta\text{ O.P.} = \underbrace{\{P_B - P_A\} + (h_B - h_A)\,\rho_A}_{\text{determined in the osmometer with a rigid semi-permeable membrane}} - \underbrace{(h'_B - h'_A)\,\rho_A}_{\text{determined in the surface tension U-tube with a flexible impermeable membrane}}$$

EXPERIMENTAL

Osmometer

The general design of the osmometer is given in Fig. 2, and the detailed construction of the glass parts in Fig. 3.

The central tube, A, carries the semi-permeable collodion sac and is filled with protein solution so that the meniscus lies in the parallel part, C, of the central tube (int. diam. 8 mm.). The main body of the osmometer, F, is filled with NaCl solution against which the protein solution has been previously dialysed, and the meniscus between air and NaCl solution in the sidearm, D, is arranged to fall in the uniform part of this tube at about the same level as the protein-solution meniscus in C. The ground joint between A and the osmometer body is greased with rubber grease and sealed with mercury. At E and G are two further ground joints which enable the osmometer to be connected by a glass capillary tube (diam. about 1 mm.) to one limb, H, of the manometer U-tube. To obtain flexibility this tube is made in two parts joined by polythene tubing. The manometer limbs are made from precision bore Veridia tubing (Chance Bros.) of 2 mm. int. diam. The limb I is open to the air.

Two manometric fluids were used, water and bromoform (density, relative to water at 25°, 2·886). Fig. 4 shows in detail the pressure control arranged for bromoform, which attacks rubber. The manometer levels are measured with a cathetometer to 0·001 cm.

The frame of the osmometer is arranged to lie in one plane so that when the apparatus is moved across, by sliding its supporting clips along the top of the thermostat bath, the levels of the menisci in the osmometer, in the surface tension U-tube mounted alongside and in the manometer can be measured with the same cathetometer. In this way the accurate levelling of the cathetometer cross wire against the undisturbed water level of the thermostat is not altered. All glass parts are attached to the frame by securing them against projections with rubber bands. This facilitates dismantling for cleaning.

A 0·3 mm. capillary, B, containing diffusate, connects the main body of the osmometer to the limb D. In this capillary is the indicator droplet, movement of which gives

a measure of the flow along the capillary, and hence across the membrane.

In constructing the glass parts, several (4) central tubes, tube *D*, and the surface tension U-tube were all made from the same length of Veridia tubing to ensure uniform internal diameter (8 mm.).

ability to obtain a value for the zero-flow pressure to ±0·1% of its value; (*c*) impermeability to the protein used (mol.wt. about 37000) as judged by the absence of cloudiness when some of the diffusate, taken from the main body of the osmometer after an experiment, was mixed with an equal volume of 25% (w/v) trichloroacetic acid solution and

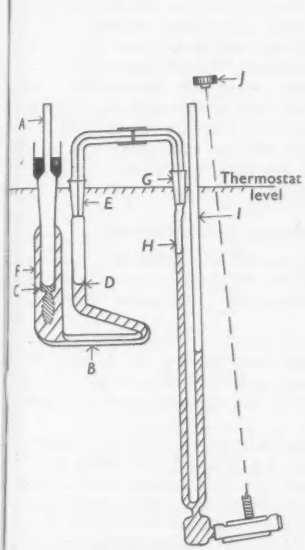

Fig. 2. General design of osmometer.

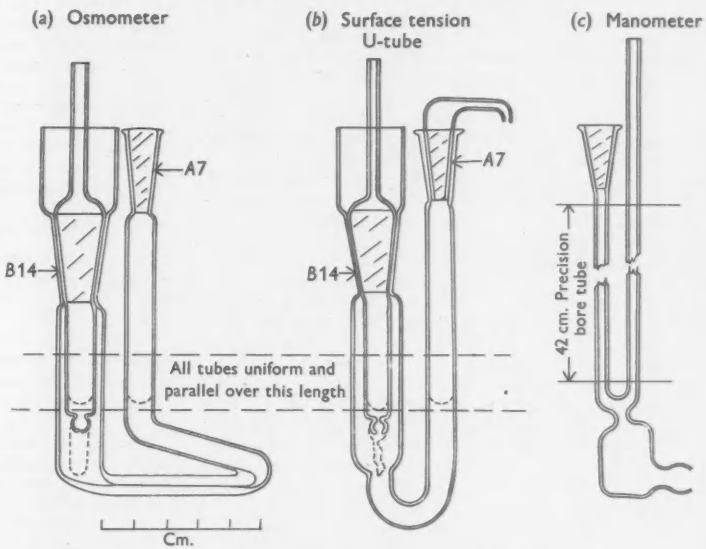

Fig. 3. Detailed design of glass parts.

Indicator droplet. The indicator droplets are prepared by adding a few drops of vacuum-distilled *o*-chlorotoluene to about 20 ml. of diffusate. Slight shaking disperses the drops into a number of droplets, and a droplet of the optimal size, a little less than the capillary diameter, can be selected by means of a Pasteur pipette, judging the size of the droplet by eye—or by allowing it to flow through a 0·3 mm. capillary and observing its shape. The droplets are less liable to stick if prepared at least 2 days before use. A 'stock' was often used for several weeks.

The capillary has to be perfectly clean and hydrophilic for the droplet to remain free. HNO_3 : ethanol is used to remove all grease, and ethanolic KOH is then left in contact with the capillary for at least 1 hr. to render the glass hydrophilic. In washing with distilled water and diffusate, during the setting up, no air-solution interfaces should be drawn along the capillary, since grease accumulates at such surfaces. Movement of the droplet is timed by observation with a telescope containing two vertical cross-wires having a separation equivalent to about twice the apparent diameter of the capillary in the telescope field.

Membranes

A new technique was developed to make membranes which were able to meet the standards essential for the method. These standards are: (*a*) ability to support an adequate pressure (most would support 50 cm. of mercury); (*b*) adequate permeability to solvent, as judged by the

allowed to stand. This test proved more sensitive than control ultrafiltration tests on the membrane.

The principle of the method is to make a suitable mandrel on the end of the central tube from the osmometer, to coat

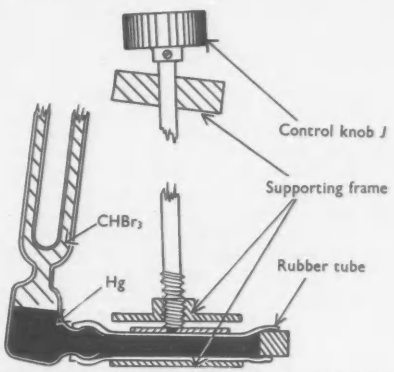

Fig. 4. Method of adjusting the bromoform manometer.

this with collodion and allow it to dry. After removal of the mandrel material the membrane is in its final position, and has at no stage been handled.

The mandrel. The central tube of the osmometer is cleaned with HNO_3 : ethanol, washed and dried. A solution of supersaturated $Na_2S_2O_3.5H_2O$ is prepared from thiosulphate crystals by fusion and boiling off about 5% of the water of crystallization. A piece of semi-transparent polythene tube about 0·5 cm. bore and 2·5 cm. long is washed, dipped in undiluted 'Teepol' (a liquid detergent, Shell Chemicals Ltd.), rinsed slightly and dried.

This tube is closed at the bottom with a glass plug, previously dipped into the supersaturated thiosulphate in order to deposit a layer of crystals on the end. The mould so formed is then filled with the supersaturated solution (at 50–60°) ensuring the absence of air bubbles. The central tube is quickly placed into the top of the mould, displacing some of the fused thiosulphate into the end of the central tube. Crystallization from the bottom is brought about by lowering the whole slowly into iced water. When crystallization is complete the glass plug and polythene tube are removed, leaving a rod of solid $Na_2S_2O_3$ projecting from the central tube. The end of this mandrel is rounded off with emery paper, and polished on the damped palm of the hand. A careful inspection is made for any flaws or small air pockets on the surface of the mandrel. Any visible flaws are removed or the mandrel is rejected. Small crystals grown from the bottom give the best results.

The collodion coat. The collodion solution was: 60 ml. ether treated with Na metal; 40 ml. ethanol distilled from Al-Hg; 2 ml. ethylene glycol; 2 g. pyroxylin (British Drug Houses Ltd., vacuum desiccated). This solution, after preparation, was centrifuged and degassed under reduced pressure to reduce bubble formation when the mandrel was dipped into it.

In coating, the mandrel surface is wiped with an ethanol-soaked cloth and dried. The mandrel and central tube are dipped in the collodion solution to a suitable depth and rotated to ensure absence of adhering air bubbles. The mandrel is withdrawn slowly (taking 2 sec.) until the tip just touches the surface, when it is allowed to drain for a further 2 sec. It is then withdrawn completely, inverted for 5 sec. with rotation, reverted for 5 sec. and inverted once more for 5 sec. It is then kept horizontal and rotated until placed in a desiccator where it is dried horizontally whilst being rotated at about 4 rev./min.

The drying time is used to control the permeability to protein. It was found necessary to dry longer for higher protein concentrations, the times ranging from 30 to 60 min. for the range of osmotic pressures measured.

When the drying is almost complete the membrane is secured to the central tube by a rubber band wrapped round several times, and then the thiosulphate is removed by immersing the membrane in ice-cold water. When solution is complete, the membrane is well washed with distilled water, and the internal washings are removed with a fine Pasteur pipette. It is stored in distilled water with the central tube and membrane full of distilled water.

Membrane drying. Before use, the inside surface of the membrane is dried to avoid dilution of the protein solution during the setting up, and consequent disturbance of the previously established dialysis equilibrium. The drying is performed, after removal of most of the water from the inside of the membrane by pipette, by drawing a stream of air over the inside surface through a fine capillary tube connected to a water pump. The outside of the membrane is surrounded by distilled water and is kept at a lower pressure than the atmosphere, by placing the central tube and membrane in a U-tube to which a negative pressure can be applied. Drying overnight in this way does not lead to a loss of permeability.

The thermostat bath

The temperature of the osmometer must be controlled within ±0·001°. This degree of control was obtained by using a hollow cylindrical toluene-filled regulator. This regulated a 100 W light bulb, used as a heater, by means of a simple vacuum tube circuit. The stirring was vigorous and was arranged so that water from the heating bulb passed over the regulator, and so that only mixed bath contents reach the osmometer. The rate of cooling of the bath enabled the cyclical variation in temperature of the bath to be estimated at between ±0·00025 and ±0·0005° for the mixed bath contents.

Detailed procedure and setting up

The protein solutions are used after preliminary dialysis against the solvent, at 25° for 4 days, under a layer of toluene to prevent evaporation and growth of micro-organisms.

The manometer is subjected to the same cleaning procedure as the osmometer when water is the manometric fluid; when $CHBr_3$ is used, only the HNO_3 : ethanol water treatment is carried out and the manometer is dried before use.

In setting up, the manometer is first assembled and left with the liquid level at the top of tube *H*. The osmometer is filled with diffusate and put in a suitable stand. The membrane is removed from the drier after setting up a positive pressure inside it by blowing down a rubber tube attached to the top of the central tube and sealing it with a screw clip. It is placed in the osmometer, after sluicing down the outside with diffusate, and is distended by sucking on limb *D*. The osmometer is placed on the frame. The level in *D* is adjusted with a Pasteur pipette, and a suitable droplet selected from the stock with the same pipette and introduced at *E*. The connecting capillary is inserted and a negative pressure applied outside the membrane with the control knob, *J*. The whole apparatus is then set in the thermostat bath, and the droplet observed. By suitable manipulation of the apparatus during this setting up, the droplet can be caused to fall along the tube *D* to the capillary without touching a side wall. This avoids the risk of its sticking to the wall. Once it is in the capillary, sticking rarely occurs. The positive pressure inside the membrane can now be released and the pressure control manipulated to keep the droplet in the capillary. Finally the protein solution is introduced, the manometer set to keep the droplet stationary and the central tube plugged with cotton wool. It was found best to set up the apparatus in the afternoon, without mixing the protein solution in the sac, to mix late in the evening, and to determine the osmotic pressure next day.

The measurement is carried out by observing the rate of movement of the droplet alternately in either direction along the capillary, for in this way changes in composition at the membrane are minimized. One min. should be allowed between altering the pressure and making a rate measurement, to allow rapid membrane stretching to subside. The optimal rates lie between 100 and 300 sec. for the movement from one cross-wire to the next. Pressures on the manometer are read before and after each rate deter-

Table 1. *A typical record of the measurements made in determining the osmotic pressure of a solution*

(21. ii. 51. β-Lactoglobulin, approx. 5·8 g./100 ml. solution in 1·19 M-NaCl, 25·000°. Membrane, single coat, dried 40 min. Manometer fluid $CHBr_3$ set up 12.00 hr. 20. ii. 51, without mixing; mixed 10.00 hr. 21. ii. 51.)

	Level 1 (cm.)	Level 2 (cm.)	Δ (cm.)	Rate ($sec.^{-1}$)	Time (sec.)
10.55 hr.	31·422	13·758	17·664	−719	139
	31·422	13·756	17·666		
	31·682	14·848	16·834	+559	179
	31·686	14·842	16·844		
	31·484	13·968	17·516	−422	237
	31·476	13·968	17·508		
	31·724	15·026	16·698	+775	129
	31·724	15·024	16·700		
	31·492	15·040	17·452	−395	253
	31·494	14·044	17·450		
	31·706	14·934	16·772	+610	164
	31·706	14·934	16·772		
	31·454	13·854	17·600	−602	166
	31·450	13·854	17·596		
	31·740	15·124	16·616	+826	121
	31·744	15·122	16·622		
	31·504	14·082	17·422	−341	293
	31·504	14·084	17·420		
11.55 hr.	31·704	14·934	16·770	+581	172
	31·706	14·932	16·774		

Zero flow at 17·19 ± 0·01 cm. $CHBr_3$ = 49·61 ± 0·03 cm. water.

Corrections: Δ levels = +0·062 cm.
Surface tension = +0·162 cm. (1·19 M-NaCl)
Total = 1·035 × 0·224 cm. water.

Osmotic pressure = 49·61 + (1·035 × 0·224) = 49·84 ± 0·03 cm. water.

Refractometer set up 12.00 hr. 21. ii. 51. Blank = 0·0165 in.

Time (hr.)	Left (in.)	Right (in.)	Δ (in.)	Temp. (°)
12.15	0·9339	0·0503	0·8836	20·0
12.40	0·9331	0·0508	0·8823	20·0
12.50	0·9329	0·0510	0·8819	19·9
13.05	0·9329	0·0512	0·8817	19·9

Deflexion = 0·8818 − 0·0165 = 0·8653 in.

Concentration = 5·793 g./100 ml. solution ≡ 6·057 g./100 ml. solvent.

mination. A typical record sheet (Table 1) and graph (Fig. 5) show the procedure in more detail and indicate the method of plotting employed. By joining the points successively as they are obtained, any abnormalities can be detected. This is exemplified in a graph (Fig. 5) which was obtained when the thermostat failed because of mains voltage variations.

Surface tension and density corrections

In the theoretical section the basis for the determination of these corrections was outlined. The U-tube used for the determinations is shown in Fig. 3. The small right-angle tube attached to the open limb prevents contamination by dust. It was calibrated against the osmometer by measuring the levels in the limbs *C* and *D* of the osmometer and in the surface tension U-tube, using several central tubes, all being filled with water, and no membranes being present. The results indicated that any central tube could be used to measure the surface tension correction to better than 0·01 cm.

The flexible sacs were made from a vulcanized latex (labelled '60% D.R.C.') which the British Rubber Products Research Association provided. This latex was diluted with 4·5 vol. of water and the membranes prepared from the muslin-filtered solution by dipping a rounded glass mandrel, allowing it to dry for 30 min. in a vertical position, redipping and again allowing to dry, and steaming for 5 min. After washing and drying, the membranes were trimmed at 8 cm. length and rolled on themselves to about 3 cm. from the top, and then dipped into French chalk. They were then removed, unrolled to the 3 cm. length and, after thorough washing, stored in distilled water. They did not lose flexibility or impermeability in 12 months' storage. It was found that over a wide range of collapse the pressure difference required to keep these sacs collapsed was constant (e.g. 0·016 ± 0·006 cm.).

The differences in levels became constant about 1 hr. after setting up. These differences, corrected for the collapse pressure, are shown in Fig. 6.

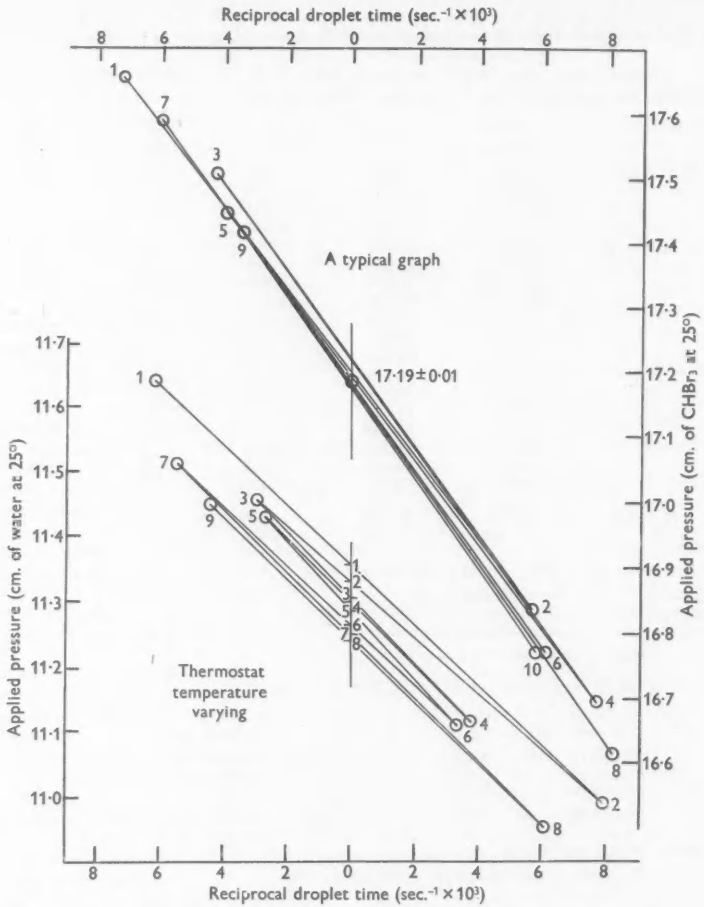
Reciprocal droplet time (sec.$^{-1}$ × 10^3)
A typical graph
17·19 ± 0·01
Thermostat temperature varying
Applied pressure (cm. of water at 25°)
Applied pressure (cm. of CHBr$_3$ at 25°)

Fig. 5. A typical graph and the graph from a determination in which the thermostat temperature was varying.

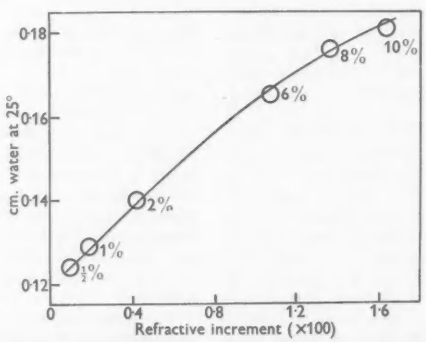

Fig. 6. Surface tension and density corrections, for various concentrations of β-lactoglobulin in 1·19 M-NaCl.

RESULTS

The results and the refractometric procedure are presented in Part 2 of this paper. All determinations carried through to completion are quoted. Each was performed on about 0·5 ml. of solution using the methods indicated, the order for each determination being: osmotic pressure, refractive increment and surface tension correction; the last was actually done on pooled samples but all three determinations can, if required, be performed on the same 0·5 ml. sample.

Part 2. Osmotic pressure measurements on isoelectric β-lactoglobulin solutions

The method described in Part 1 was designed to give results accurate enough for the investigation of deviations of protein osmotic pressures from ideality over a wide range of osmotic pressures whilst using the minimum of solution. β-Lactoglobulin was chosen for test purposes because of its ready availability in a crystalline state. The results, presented below, show that isoelectric lactoglobulin solutions show marked non-ideality which can be determined with good precision by this method.

THEORY

Calculation of mole fractions from refractive data

Let μ_{Pr} represent the refractive index of the protein against a vacuum, assumed independent of its concentration (cf. Adair & Robinson, 1930*a*, and Perlmann & Longsworth, 1948). Let μ_S' and μ_S'' be the refractive indices of the solvent in the protein solution and in the diffusate respectively, and v_{Pr}', v_S' and v_S'' the volume fractions of the protein in its solution, and of the solvent in the protein solution and diffusate.

Then the refractive increment (R.I.) of the protein solution against the diffusate is given by

$$\text{R.I.} = \mu_{Pr} v_{Pr}' + \mu_S' v_S' - \mu_S'' v_S'',$$

or, since $v_S' = 1 - v_{Pr}'$ and $v_S'' = 1$,

$$\text{R.I.} = (\mu_{Pr} - \mu_S') v_{Pr}' + (\mu_S' - \mu_S'').$$

For simple systems $\mu_S' = \mu_S'' = \mu_S$, but in protein-electrolyte-water systems Donnan and similar effects may make this untrue. However, if the 'solvent' (water plus electrolyte) can be made to approach the same composition on both sides of the membrane, then $\mu_S' = \mu_S''$ and the system can be considered as a two-component system in the refractive and osmotic treatment. This condition is approached if the protein is nearly isoelectric, if the electrolyte concentration is high, and if the protein shows little preferential interaction with one component of the 'solvent'. The present system is an attempt to approach these requirements. Thus the method of preparation of the solutions is such that the lactoglobulin molecule has an estimated charge of only 4, this figure being obtained from electrophoretic experiments on similarly prepared solutions in 0·1M-sodium chloride, combined with data from the literature on the variation of chloride ion binding by proteins with increasing sodium chloride concentrations; the salt concentration is high enough to suppress the Donnan effect from such a charge; and it is chosen (1·19M-sodium chloride) so that the fractional change in the molal activity coefficient with increase in ionic strength is minimized. Because of these considerations, the system has been analysed as a two-component system. Thus

$$\text{R.I.} = (\mu_{Pr} - \mu_S) v_{Pr}'. \qquad \text{(i)}$$

Data given by Perlmann & Longsworth (1948), Kruis (1936), and Brodsky & Fillipowa (1933) and Eqn. (i) give

$$(\mu_{Pr} - \mu_S) = \frac{0{\cdot}1859}{\bar{V}} - 0{\cdot}00625, \qquad \text{(ii)}$$

where μ_S is the refractive index of 1·19M-sodium chloride, $\bar{V}$ is the specific volume of lactoglobulin, $0{\cdot}1859 \times 10^{-2}$ is the specific refractive increment of lactoglobulin in 0·5M-sodium chloride at 20° for mercury green light, and 0·00625 is $(\mu_S - \mu_{\text{0·5M-sodium chloride}})$.

Define the mole fraction n_{Pr}^* of the lactoglobulin as $N_{Pr}/(N_{Pr} + N_S)$ where the asterisk indicates that the scale refers to S, the mixed solvent 1·19M-sodium chloride, and where the N's refer to the total number of 'molecules' present.

Then, if the ϕ_V's represent apparent molar volumes, and the n's mole fractions,

$$v_{Pr}' = \frac{N_{Pr}\,\phi_V^{Pr}}{N_{Pr}\,\phi_V^{Pr} + N_S\,\phi_V^{S}} = \frac{n_{Pr}^*\,\phi_V^{Pr}}{n_{Pr}^*\,\phi_V^{Pr} + n_S^*\,\phi_V^{S}}.$$

But $\phi_V^S \ll \phi_V^{Pr}$ and $n_S^* = 1 - n_{Pr}^*$,

therefore

$$n_{Pr}^* = \frac{\phi_V^S}{\phi_V^{Pr}\left(\dfrac{1}{v_{Pr}'} - 1\right)}. \qquad \text{(iii)}$$

Writing ϕ_V^{Pr} as $M_{Pr}\,\bar{V}$, combining Eqns. (i), (ii) and (iii) and substituting 0·7514 for $\bar{V}$ at 20° (Pedersen, 1936) gives

$$n_{Pr}^* = \frac{\phi_V^S}{M_{Pr}}\left\{\frac{0{\cdot}1812}{\text{R.I.}} - 0{\cdot}7514\right\}^{-1}. \qquad \text{(iv)}$$

Any uncertainty in the value for $\bar{V}$ does not greatly affect n_{Pr}^*; thus a 1% error in $\bar{V}$ never causes an error > 0·1% in n_{Pr}^* as determined refractometrically.

It is to be noted that $0{\cdot}1812 \times 10^{-2}$ is the effective specific refractive increment of the lactoglobulin

under these conditions, that $100\Big/\left\{\frac{0{\cdot}1812}{\text{R.I.}}\right\}$ gives the concentration, c, of lactoglobulin in g./100 ml. of solution, and that $100\Big/\left\{\frac{0{\cdot}1812}{\text{R.I.}}-0{\cdot}7514\right\}$ gives the concentration, m, in g./100 ml. of solvent. Thus

$$n^*_{Pr}=\frac{\phi^s_V}{M_{Pr}}\frac{m}{100}. \qquad \text{(v)}$$

Osmotic pressure equations and non-ideality

If the mixed solvent is treated as a single component, then the osmotic pressure equation can be written (cf. Bjerrum, 1918):

$$\pi\overline{V}^*_s=-g^*\boldsymbol{RT}\ln n^*_s=-g^*\boldsymbol{RT}\ln(1-n^*_{Pr})$$

the asterisks again indicating this arbitrary choice of components, g^* being the osmotic coefficient, and $\overline{V}^*_s$ the partial molar volume of the 'solvent'.

But n^*_{Pr} is always less than 0·0001, so that

$$\pi\overline{V}^*_s=g^*\boldsymbol{RT}n^*_{Pr},$$

whence, from (v),

$$\pi=g^*\frac{\boldsymbol{RT}}{M_{Pr}}\frac{\phi^s_V}{\overline{V}^*_s}\frac{m}{100}.$$

$\phi^s_V=18{\cdot}05$ ml. at 20·5°, $\overline{V}^*_s=18{\cdot}10$ ml. at 25° (calculated from data given by Harned & Owen, 1950) and $\boldsymbol{RT}$ at 25° in the units used (pressure in cm. water, and $\overline{V}^*_s$ in ml.) is $2{\cdot}534\times10^7$.

Hence

$$\pi=g^*\frac{m}{M_{Pr}}2{\cdot}527\times10^5. \qquad \text{(vi)}$$

If the system is ideal $g^*=1$ at all values of m, if it is not ideal the variation of g^* with m enables the non-ideality to be calculated. This non-ideality will appear as a non-linear term in a π against m plot.

EXPERIMENTAL

Preparation of solutions. The solutions were prepared from lactoglobulin made by the method of Cecil & Ogston (1949), modified by the author in conjunction with R. Cecil.

The casein was removed from about 7·5 l. of skimmed milk by filtration, after precipitation with 392 g. of $(NH_4)_2SO_4$/l. To the filtrate were added a further 124 g./l. of $(NH_4)_2SO_4$. The crude lactoglobulin was removed next day by filtration, and dissolved in about 100 ml. of water. Dialysis against water was carried out until the concentration of $(NH_4)_2SO_4$ in the diffusate was less than 0·2 g./100 ml. After centrifuging at 15 000 rev./min. for 45 min. 48·8 g./100 ml. of A.R. $(NH_4)_2SO_4$ were added to the supernatant. The precipitate was collected by centrifugation and washed by stirring with a similar (48·8 g./100 ml.) solution of $(NH_4)_2SO_4$, followed by centrifugation and decantation. It was dissolved in about 60 ml. of water and, after centrifuging, dialysed against two changes of 0·1 M-sodium acetate buffer saturated with toluene, pH about 5·2, and then against several changes of water. The resulting oil was collected and seeded (scratching the side of the glass vessel and keeping at room temperature help to promote crystallization). The crystals obtained were well washed with water and recrystallized from 1·0 M-NaCl. Electrodialysis of a suspension of the resulting crystals was carried out to remove remaining traces of salt, using an apparatus designed to avoid contamination of the protein with electrode reaction products. It was continued until the current was constant and until no change occurred when the crystals were stirred.

The electrodialysed crystals were freeze-dried and showed no change in solubility, crystallizing ability, sedimentation constant (Svedberg ultracentrifuge) and diffusion constant (Gouy diffusiometer), or of the apparent homogeneity, after the freeze-drying.

Several solutions of the lactoglobulin were prepared in 1·19 M-NaCl, dialysed for 4 days at 25° under toluene, and stored in sealed tubes at 2° until required. No signs of deterioration were observed in the 3 months' period of storage and use.

Refractive-increment determinations. The refractive increments of the contents of the osmometer sac were determined in a differential refractometer (Cecil & Ogston, 1951) against diffusate from the main body of the osmometer. In this way a correction for any protein which has leaked through the membrane is automatically applied. Before the membrane drying times were known, the concentration of protein leaked through might reach, but never exceeded, 0·1% of the internal concentration; later, the leakage was much less, so that this correction was never significant.

The refractometer was calibrated against two KCl solutions, and its temperature was $20{\cdot}5\pm0{\cdot}75°$ for all determinations. For protein solutions up to 6·5 g./100 ml. one deflexion measurement and a blank for the whole series was required. Above this concentration a stepwise procedure was employed:

Solutions	Instrument reading
Lactoglobulin against NaCl (12 g./100 ml.)	x
NaCl (12 g./100 ml.) against osmometer diffusate	y
NaCl (12 g./100 ml.) against NaCl (12 g./100 ml.) (blank)	p
Diffusate against diffusate (blank)	

The refractive increment is then

$$\{(x+y)-(p+q)\}\times\text{cell constant}.$$

It was found that p, q and y need be determined only once, as y was found to be constant to $\pm0{\cdot}1$%; subsequently any refractive increment could be obtained with a single deflexion measurement.

RESULTS AND DISCUSSION

The experimental results are quoted in Table 2. In it are also given the concentrations of the lactoglobulin in g./100 ml. of solution (c), and in g./100 ml. of solvent (m), calculated as described. The surface tension and density corrections which were used in obtaining the osmotic pressures are shown in Fig. 6. Finally the osmotic pressure results are plotted against m in Fig. 7. The smooth curve corresponds to the statistically fitted regression and the straight line to the initial slope obtained from this regression.

Table 2. *Summary of determinations of the the osmotic pressures of solutions of β-lactoglobulin*

π (cm. water)	Refractive increment × 100	Lactoglobulin concentration (g./100 ml. solution)	Lactoglobulin concentration (g./100 ml. solvent)	π/c	π/m
7·04	0·1834	1·012	1·020	695·6	690·3
7·37	0·1900	1·049	1·057	702·6	697·1
7·56	0·1967	1·086	1·095	696·1	690·5
7·58	0·1949	1·076	1·085	704·5	698·8
16·94	0·4106	2·266	2·305	747·6	734·9
17·22	0·4095	2·260	2·299	762·0	749·1
17·64	0·4128	2·278	2·317	774·4	761·2
17·72	0·4264	2·353	2·395	753·1	739·8
18·54	0·4512	2·490	2·537	744·6	730·7
32·81	0·7473	4·124	4·255	795·6	771·0
33·96	0·7532	4·157	4·290	817·1	791·6
33·96	0·7556	4·170	4·305	814·4	788·9
34·81	0·7784	4·296	4·439	810·4	784·3
49·84	1·0496	5·793	6·057	860·2	822·9
50·78	1·0727	5·920	6·196	857·7	819·6
51·24	1·0814	5·968	6·246	858·8	820·4
66·42	1·3299	7·340	7·770	904·6	854·8
67·93	1·3469	7·434	7·874	913·7	862·7
69·69	1·3707	7·565	8·019	921·3	869·0
69·74	1·3757	7·592	8·052	918·5	866·2
70·45	1·3834	7·635	8·097	922·9	870·1
83·62	1·5838	8·741	9·355	956·6	893·9
86·57	1·6248	8·967	9·615	965·3	900·3
91·51	1·6832	9·290	9·990	984·6	916·0

The statistical examination of the results was carried out as follows: The 'best' curved regression formula was fitted to the points of the π against m plot by the method of least squares; a second-order regression was most satisfactory, the third order giving no significant improvement in fit. Linear regressions were fitted to the results expressed as π/m against m, and π/c against c plots. The expressions obtained and the standard errors of the constants follow:

$$\pi = 6{\cdot}83(\pm 0{\cdot}10 \text{ or } 1{\cdot}5\%)\, m + 0{\cdot}228(\pm 0{\cdot}0094 \text{ or } 4{\cdot}1\%)\, m^2. \quad \text{(vii)}$$

$$\pi/m = 6{\cdot}79(\pm 0{\cdot}18 \text{ or } 2{\cdot}7\%) + 0{\cdot}233(\pm 0{\cdot}0065 \text{ or } 2{\cdot}8\%)\, m. \quad \text{(viii)}$$

$$\pi/c = 6{\cdot}72(\pm 0{\cdot}18 \text{ or } 2{\cdot}6\%) + 0{\cdot}327(\pm 0{\cdot}0068 \text{ or } 2{\cdot}1\%)\, c. \quad \text{(ix)}$$

The standard errors indicate that the most satisfactory method of obtaining a molecular weight from these data is to fit a curved regression, and to use the linear coefficient to calculate a molecular weight, rather than to use the customary π/m and intercept method. This is because the intercept lies outside the experimental range of points and so is statistically less precisely known. Using results obtained over a wide range of concentration in a non-ideal system also has two advantages over using data at low concentrations: (*a*) the measurements are usually more precise at higher osmotic pressures; (*b*) a slight positive non-ideality at low concentration may easily be overlooked and will give a value for the molecular weight which is too small.

Combining Eqns. (vi) and (vii), and recollecting that $g^* \to 1$ as $m \to 0$, one obtains a value for M_{Pr} of 37000 ± 540 for this sample of β-lactoglobulin. The non-linear term in Eqn. (vii), or the linear term in (viii) enable the non-ideal osmotic pressure to be expressed in terms of m. Thus from (viii)

$$\pi_{\text{non-ideal}} = 0{\cdot}233(\pm 0{\cdot}0065)\, m^2.$$

(The value of π_{ideal}, from (viii), is $6{\cdot}79m$.)

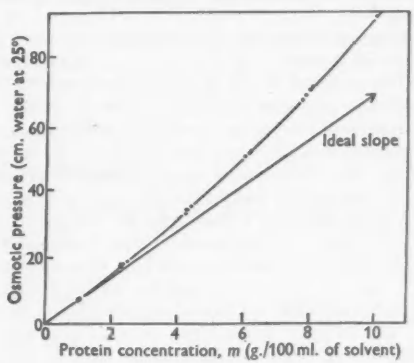

Fig. 7. Osmotic pressure against concentration, for solutions of isoelectric β-lactoglobulin in 1·19 M-NaCl.

Thus the non-ideal partial molar free energy of dilution of lactoglobulin in these solutions, $\left(\frac{\partial G}{\partial n_s^*}\right)^{25°}_{\text{non-ideal}}$ is given by $\pi_{\text{non-ideal}}\,\bar{V}_s^*$ expressed in the correct units (cal./mole of solvent), i.e.

$$\left(\frac{\partial G}{\partial n_s^*}\right)^{25°}_{\text{non-ideal}} = 0{\cdot}985(\pm 0{\cdot}027 \text{ or } 2{\cdot}8\%) \times 10^{-4}\, m^2 \text{ cal./mole of solvent.}$$

Comparison with the ideal value shows that positive deviations up to 35% are observed at the highest concentrations considered.

The value for the molecular weight obtained agrees satisfactorily with most previous osmotic work: Gutfreund (1945), 37 800 ± 900; Johnston & Ogston (1946), 37 300 ± 300; though Bull & Currie (1946) give a lower value 35 020 ± 140 and Christensen (1952) higher values 38 750 and 39 730. X-ray and ultracentrifugal values range from 33 000 to 42 000. Discussion of these differences is given by Cecil & Ogston (1949) and Bull & Currie (1946).

The large deviations from ideality in these lactoglobulin solutions seem to have gone undetected in previous work. This is probably due to the narrow range of concentrations covered by previous workers, none having made measurements above 4 g./100 ml. Such deviations may be due to marked interaction with the solvent, repulsive forces between the protein molecules, or to entropy effects due to the large size of the protein molecules. An investigation at other temperatures would help distinguish between these possibilities, but the great solubility of lactoglobulin in neutral salt solutions suggests that 'solvent' interaction is marked.

Accuracy of method. The standard error of the determined osmotic pressure from the fitted regression at its mean is ±0·75%, which represents the maximum probable experimental error. Much of the scatter of the points is probably due to small pH changes of variable extent brought about during the setting up. Thus earlier trials showed that it is possible to obtain values for the osmotic pressure of a given solution which agree to ±0·1% if the measurements are repeated without reassembling the apparatus, even though made on different days. The use of buffered solutions would therefore probably increase the reproducibility considerably.

Unfortunately, few workers have made measurements over wide concentration ranges, under similar conditions for each measurement, with which to compare the accuracy of the present method (Scatchard, Batchelder & Brown (1946) and Masson & Melville (1949) used variable conditions for many of the determinations), or else the results are obviously of too low accuracy to be worth analysis.

The results of three groups of workers have been selected for comparison. In making these comparisons, all the results quoted as reliable by the respective authors are used and are compared with a similar number of the present results, covering if possible the same range of osmotic pressures but selected randomly. For ease of comparison π/m plots are used. Adair & Robinson (1930*b*) give twenty-seven results obtained with an equilibrium method on buffered serum-albumin solutions; Gutfreund (1949) gives fifteen equilibrium measurements on buffered human-haemoglobin solutions; and Güntelberg & Linderstrøm-Lang (1949) give eight (nine made, but one excluded) determinations of the osmotic pressure of unbuffered plakalbumin solutions made with a dynamic osmometer. Christensen (1952) has made measurements of similar precision on solutions of β-lactoglobulin, by the method of Güntelberg & Linderstøm-Lang (1949).

The results of a least-square treatment of these data are shown in Table 3, the regression fitted being

$$\pi/m = a + bm.$$

Table 3. *Statistical comparison of the accuracy of some published measurements of osmotic pressure with the present measurements*

(Percentage accuracy of the estimates of coefficients in the equation $\pi/M = a + bM$.)

Authors	No. of observations	Standard errors of the mean (%) a	b
Adair & Robinson (1930*b*)	27	7·7	5·3
Present work	24	2·7	2·8
Güntelberg & Linderstrøm-Lang (1949)	8	2·0	8·1
Present work	8	2·3	6·8
Christensen (1952) (series B)	10	4·3	15·2
Present work	10	3·4	22
Gutfreund (1949)	15	7·7	23
Present work	15	3·3	7·2

The table shows that the present method is capable of a considerably greater precision than either of the two equilibrium methods quoted. The differences between Güntelberg & Linderstrøm-Lang's and Christensen's results and those of the author are not significant, but the apparatus used by these workers requires about 10 times the volume of solution which is used in the present apparatus.

SUMMARY

Part 1

1. The theory for the dynamic osmometer developed in this work for the study of non-ideality in protein solutions is presented.

2. The general design and details of the essential parts of the osmometer are described. The osmometer requires less than 0·5 ml. of solution per measurement.

3. A new method for making thin collodion membranes has been developed, by which membranes of high permeability to the solvent but completely impermeable to lactoglobulin can be made.

4. The working procedure is described.

5. A new method is given for obtaining the surface tension and density corrections to be used in osmometry. The osmotic pressure, surface tension correction and refractive increment can all be measured on a single 0·5 ml. sample.

Part 2

1. The calculation of mole fractions from refractometric data, and the analysis of the data in terms of a two-component system are discussed.

2. The preparation of the β-lactoglobulin and solutions is described.

3. The refractometric procedure is outlined.

4. The results obtained by the methods described on unbuffered lactoglobulin solutions are presented and analysed. The solutions show a marked degree of non-ideality (up to 35% at a 10 g./100 ml. protein concentration). The methods of calculation of a molecular weight from such data are considered, and a value for this lactoglobulin of $37\,000 \pm 540$ is obtained, this being calculated from twenty-four measurements covering a range of concentrations from 1 to 10 g./100 ml. protein.

5. The results are compared with previous work, and it is concluded that the present method is considerably more precise (and capable of improvement by the use of buffered solutions) than the equilibrium methods with which it is compared. It is as precise as another dynamic method considered, but requires only one-tenth of the volume of solution.

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Studies on Cholinesterase

9. SPECIES VARIATION IN THE SPECIFICITY PATTERN OF THE PSEUDO CHOLINESTERASES

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As a result of their studies on the relative substrate specificities of various cholinesterase preparations, Mendel and co-workers concluded that vertebrate tissues contain two distinct types of enzymes capable of hydrolysing acetylcholine *in vitro*, both of which are sensitive to inhibition by low concentrations of eserine (Mendel & Rudney, 1943; Mendel, Mundell & Rudney, 1943). These two types were designated originally as true cholinesterase and pseudo cholinesterase (Mendel & Rudney, 1943).

Subsequent investigations have shown that true cholinesterases of human erythrocytes, horse erythrocytes and pigeon brain (Adams, 1949; Mounter & Whittaker, 1950; Whittaker, 1949) are most active towards those choline and non-choline esters whose structure approaches most closely

to that of acetylcholine. The pseudo cholinesterases of human serum and horse serum, on the other hand, exhibit their highest activity towards those esters which show a close structural resemblance to butyrylcholine (Adams & Whittaker, 1949*a*; Sturge & Whittaker, 1950). Consequently it has been proposed that the two types of cholinesterases should be classified on the basis of this difference in specificity patterns and designated as acetocholinesterases and butyro-cholinesterases respectively (Richter, cited by Sturge & Whittaker, 1950; cf. Whittaker, 1951).

These designations seemed to be particularly suitable from an enzymological point of view, whether or not they will prove to be related to the distinct physiological functions of the two types of cholinesterases. However, more recent investigations by Ord & Thompson (1951) have shown that rat heart ventricle contains a pseudo cholinesterase which acts most rapidly upon propionylcholine. These authors did not determine whether this difference was due to the species or to the tissue used, but it seems to justify the classification of this enzyme as a propiono-cholinesterase.

This finding suggested that a more extensive investigation of the substrate specificity patterns of pseudo cholinesterases from different species would be justified. It has been emphasized by Hawkins & Mendel (1946) and Mendel & Hawkins (1950) that the ratio of the maximal activities of a cholinesterase towards two different substrates may vary considerably from one species to another. Similar variations have been demonstrated by Augustinsson (1949), Sturge & Whittaker (1950) and Myers (1952*a*). The present investigation was designed, in part, to study these variations in specificity patterns in greater detail, and to determine whether the propiono-cholinesterase studied by Ord & Thompson (1951) was typical of the particular species used, or whether different enzymes are found in other tissues of the same animal.

A preliminary report on this work has been presented (Myers & Mendel, 1952).

METHODS

Estimation of esterase activity. As in previous investigations (Mendel & Rudney, 1943; Hawkins & Mendel, 1949; Myers, 1952*a*), the activity of the cholinesterases was measured manometrically by the Warburg method at pH 7·4 and 37·5° in a medium containing 0·025 M-$NaHCO_3$ and saturated with 5% CO_2 in 95% N_2 (v/v). The volume of enzyme preparation was selected so as to give an activity between 60 and 180 μl. CO_2 in 30 min. as far as was practicable. The enzyme preparation was placed in the main compartment of the Warburg vessel, the substrate in the side arm, and the enzyme inhibitor when used was added to the enzyme-bicarbonate mixture in the main compartment. After equilibration of the vessels at 37·5° for about 30 min., the substrate was tipped in from the side arm. Readings were started 2 min. later and repeated at appropriate intervals, usually every 3 or 5 min., for 30–40 min. Esterase activity was calculated from the initial constant rate of hydrolysis of substrate after corrections for spontaneous hydrolysis of the ester.

Substrates. Esters tested as substrates for the various pseudo cholinesterases were acetylcholine chloride and perchlorate (ACh), propionylcholine chloride and perchlorate (PrCh), butyrylcholine chloride and perchlorate (BuCh), acetyl-(±)-β-methylcholine chloride (MCh) and benzoylcholine chloride (BCh). Most of the data on relative rates of hydrolysis of propionylcholine and butyrylcholine were determined with the perchlorate salts of these esters. In confirmation of a communication from Prof. R. H. S. Thompson, no significant difference in rate of hydrolysis was noted when a perchlorate salt was used in place of chloride. Tributyrin was also tested as substrate in some experiments; it was suspended with 5% gum acacia and diluted 50-fold in the Warburg vessel to give a final concentration of 0·2% tributyrin with 0·1% gum acacia.

Routine estimations of pseudo cholinesterase and true cholinesterase activities were carried out with benzoylcholine and acetyl-β-methylcholine in final concentrations of 0·006 M and 0·03 M respectively (Mendel *et al.* 1943). Acetylcholine, propionylcholine and butyrylcholine were tested at a final concentration of 0·045 M, unless otherwise stated; the 0·3 M stock solutions of these esters were used since the perchlorate salts were not soluble in higher concentrations. The total volume of fluid in the Warburg vessel was limited to 2·0 ml. in these cases to keep the amount of ester necessary for the determinations to a minimum; when lower concentrations of substrate were used, the fluid volume was increased to 5·0 ml. (cf. Myers, 1952*b*). The relative activity of the pseudo cholinesterase towards these esters is expressed as a percentage of the activity towards acetylcholine (Ord & Thompson, 1950, 1951).

Inhibitors. The inhibitors used were physostigmine salicylate (eserine), the dimethylcarbamate of (2-hydroxy-5-phenyl)benzyltrimethylammonium bromide (Nu 683; Hawkins & Gunter, 1946), and di*iso*propyl phosphonofluoridate (di*iso*propyl fluorophosphonate, DFP).

Esterase preparations. Horse serum and human serum were dialysed and tested without any further treatment since these sera contain large amounts of pseudo cholinesterase and only insignificant amounts of true cholinesterase (Mendel *et al.* 1943). Unless the serum preparation is dialysed (Myers, 1950, 1952*b*), or the medium itself contains high concentrations of salts (Nachmansohn & Rothenberg, 1945; Augustinsson, 1949), the variations in salt concentration with different amounts of serum may cause appreciable errors in the measured ratio of activities towards two substrates.

Mouse and rat serum were obtained from mature female animals since these sera contain larger amounts of pseudo cholinesterase than the sera from male mice and male rats respectively. The sera were fractionated with $(NH_4)_2SO_4$ as follows. A saturated solution of $(NH_4)_2SO_4$ was added dropwise to the serum with continuous stirring until the mixture reached 0·45 saturation after 40 min. (cf. Hawkins & Gunter, 1946). After standing for a further 60 min. at room temperature with occasional stirring, the mixture was centrifuged and the pseudo cholinesterase precipitated from the supernatant by adding solid $(NH_4)_2SO_4$ to approximately 0·85 saturation. This precipitate was redissolved in

half the original volume of water and dialysed overnight. This treatment was usually sufficient to reduce the true cholinesterase activity to a small percentage of the total activity towards acetylcholine. If a significant amount of true cholinesterase activity still remained in the solution, the same procedure was repeated using 0·5 saturation with $(NH_4)_2SO_4$ to precipitate the true cholinesterase. The degree of purification of the pseudo cholinesterase from the original serum was not very large but was sufficient to eliminate the only other enzyme in the serum capable of hydrolysing choline esters.

Among the mammalian species studied was the Syrian hamster; the pseudo cholinesterase activity of the serum and tissues was extremely low. Thus it was not possible to obtain accurate results with this serum; the approximate results obtained are included because this enzyme also appeared to be a propiono-cholinesterase.

RESULTS

Enzymological characteristics of the serum pseudo cholinesterases

With each preparation of pseudo cholinesterase, the concentration of Nu 683 required to produce 50% inhibition of the enzyme activity was estimated in the manner described previously (Myers, 1952*a*). The measured values are summarized in Table 1; all of these enzymes are inhibited by very low concentrations of Nu 683, although the K_I values vary between 6×10^{-10} and 3×10^{-8} M. There seems to be a tendency for the K_I values to fall into three groups with values of about 7×10^{-10}, 3×10^{-9} and 2×10^{-8} M-Nu 683, but the series given is too small to allow an evaluation of this apparent tendency. An accurate estimation of the molar concentration of enzyme by the use of Nu 683 (cf. Myers, 1952*a*) can only be obtained with those pseudo cholinesterases in the first of the three groups.

All of these cholinesterase preparations exhibited their maximal activity towards acetylcholine at high concentrations of ester. With some of the preparations, an attempt was made to estimate the concentration of acetylcholine required to give 50% of the maximal activity; a plot of activity against the logarithm of the substrate concentration gave the sigmoid curves expected (cf. Goldstein, 1944; Myers, 1952*b*). The measured values of the Michaelis constant K_s were all of the same order of magnitude, varying from about 3×10^{-4} to $1{\cdot}6 \times 10^{-3}$ M (Table 1). A substrate concentration of 0·045 M was selected as the most convenient for further investigations; this concentration is similar to that recommended by Mendel *et al.* (1943) and, since the pseudo cholinesterase activity is about 98% of the theoretical maximum in this range of concentrations (cf. Goldstein, 1944), small alterations in substrate concentration have very little effect upon the activity.

Table 1 also shows the variation in the relative activities of the different serum pseudo cholinesterases towards 0·006 M-benzoylcholine, the values given being expressed as a percentage of the activity towards 0·045 M-acetylcholine and also as a percentage of the activity towards 0·045 M-butyrylcholine. These results seem to confirm the hypothesis that the activity of the pseudo cholinesterases towards benzoylcholine is more closely related to the activity towards butyrylcholine than to the activity towards acetylcholine (cf. Adams & Whittaker, 1949*a*). As a rough approximation, the average pseudo cholinesterase activity towards butyrylcholine might be taken as seven times that towards benzoylcholine under the experimental conditions; the value for the pseudo cholinesterase of chicken serum is exceptional.

Table 1. *Enzymological characteristics of the pseudo cholinesterases from the sera of different species*

(The K_I values with Nu 683 for the pseudo cholinesterases from the sera of dog, man, horse, rat and mouse are taken from the data given previously (Myers, 1952*a*, 1952*c*). The relative activities towards 0·006 M-benzoylcholine are expressed as percentages of the pseudo cholinesterase activities towards 0·045 M-acetylcholine and towards 0·045 M-butyrylcholine. Experimental methods are described in the text.)

	Michaelis constants		Activity ratios	
Species	K_s ($\times 10^{-3}$ M-acetylcholine)	K_I ($\times 10^{-3}$ M-Nu 683)	$\frac{BCh}{ACh} \times 100$ (%)	$\frac{BCh}{BuCh} \times 100$ (%)
Dog	0·5	3·2	62	23
Man	1·4	0·9	36	17·5
Horse	1·6	0·7	30	12
Cat	—	3·6	28	12·5
Ferret	—	3·6	28	17
Duck	—	4·5	26	16
Rat	0·5	3·1	24	18·5
Pig	0·9	22	18	14
Pigeon	0·3*	27	17	13
Squirrel	—	1·5	15	10
Mouse	1·4	0·6	11	14
Chicken	0·3*	25	7	8

* Approximate values.

The activity ratios given in Table 1 for the pseudo cholinesterases of dog, horse, cat and human serum can be compared with the values calculated from the corresponding data given by Mendel *et al.* (1943), Hawkins & Mendel (1947), Adams & Whittaker (1949*a*), Sturge & Whittaker (1950), and Myers (1952*a*). This shows a good agreement between the present results and those of other investigators; there can be little doubt that these values are highly characteristic of the particular pseudo cholinesterases concerned. Furthermore, no significant differences in the ratio of cholinesterase activities towards different substrates are observed upon purification of the cholinesterase concerned

(Mendel & Rudney, 1943; Adams & Whittaker, 1949*b*; Augustinsson, 1948, 1949; Ord & Thompson, 1951), nor were any differences observed in the specificity pattern of the pseudo cholinesterase of human serum from one individual to another.

Substrate specificity patterns

Comparative specificity patterns obtained with pseudo cholinesterases from the sera of thirteen different warm-blooded species are shown in Fig. 1. Eight of these hydrolysed butyrylcholine more rapidly than any of the other choline esters tested, while four hydrolysed propionylcholine most rapidly. The first eight enzymes might be classified as butyro-cholinesterases and the latter four as propiono-cholinesterases; however, there is no clear-cut distinction between these two groups. When the patterns are arranged into a series as in Fig. 1, a gradual transition from the one extreme to the other can be seen. We have as yet not found any pseudo cholinesterase which exhibits maximal activity towards acetylcholine, but the results suggest the possibility that a pseudo cholinesterase of this type may exist.

The substrate specificity pattern of the pseudo cholinesterase from rat serum is practically identical with that reported by Ord & Thompson (1951) for the cholinesterase from rat heart. There does not seem to be any special significance in the order of the species as given in Fig. 1. The pseudo cholinesterases from related species such as hamster, rat and mouse do show somewhat similar specificity patterns, but the pseudo cholinesterase of the red squirrel exhibits a dissimilar pattern.

The pseudo cholinesterase of pig serum

Levine & Suran (1950) have reported that pig serum contains a new type of cholinesterase which hydrolyses acetylcholine fairly rapidly but which does not exhibit any significant activity towards either acetyl-β-methylcholine or benzoylcholine; this enzyme, like the pseudo cholinesterases of other species, was found to be sensitive to inhibition by 10^{-4}M-pontocaine. In the present investigation it was found that dialysed pig serum does hydrolyse 0·006M-benzoylcholine at about 18% of the rate of hydrolysis of 0·045M-acetylcholine (Table 1). On plotting the activity towards acetylcholine against the logarithm of the molar concentration of acetylcholine, a typical S-shaped curve is obtained with a K_s value of the same order of magnitude as that found with other well known pseudo cholinesterases (Table 1). The activities towards acetylcholine and benzoylcholine were both inhibited by 10^{-5}M-eserine, and the serum exhibits moderately high activity towards butyrylcholine, an ester which may also be used as a 'specific' substrate for the pseudo cholinesterases (Cohen, Kalsbeek & Warringa, 1949).

This enzyme does exhibit an exceptional substrate specificity pattern; both acetylcholine and butyrylcholine are hydrolysed more rapidly than propionylcholine (Fig. 1). However, the activities towards all of these esters were inhibited to the same degree by various concentrations of DFP and Nu 683 (Table 2), and all were decreased to the same extent when the pseudo cholinesterase was partially inactivated by heating the serum at 55°

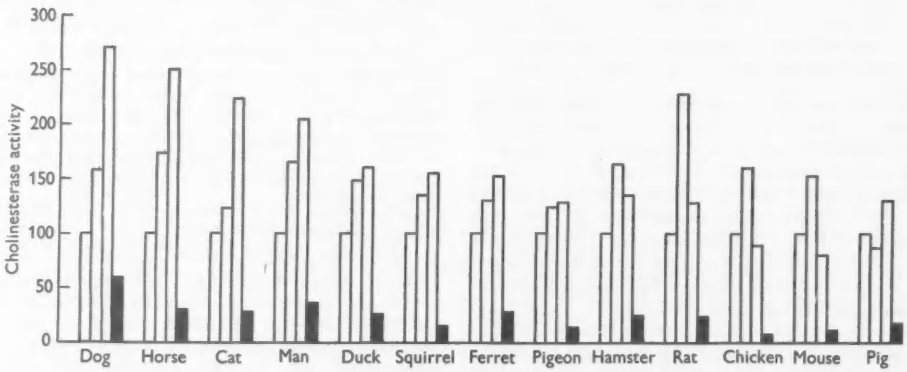

Fig. 1. Substrate specificity patterns of the pseudo cholinesterases from the sera of different species. In each case the first column indicates the relative rate of hydrolysis of 0·045M-ACh, given an arbitrary value of 100. The second column represents the relative activity of the pseudo cholinesterases towards 0·045M-PrCh, the third column that towards 0·045M-BuCh, while the fourth, in black, represents the relative activity towards 0·006M-BCh. Most of the pseudo cholinesterase preparations were obtained from their respective sera by fractional precipitation between 0·50 and 0·85 saturation with $(NH_4)_2SO_4$ as described in the text.

Table 2. *Inhibition by DFP and by Nu 683 of the hydrolysis of choline esters by pig serum*

(The pseudo cholinesterase preparation used was separated from pig serum as described in the text; this preparation showed an activity of 186 μl. CO_2/ml./20 min. towards 0·045 M-BuCl. The relative rates of hydrolysis of ACh, PrCh, BuCh and BCh are indicated in Fig. 1. The percentage inhibition of the activity towards each of these esters was measured after incubation of 1 ml. solution in a fluid volume of 5 ml. for 30 min. at 37·5° with various concentrations of DFP and Nu 682.)

Inhibition by DFP					Inhibition by Nu 683				
DFP concentration ($\times 10^{-7}$ M)	Percentage inhibition of the activity towards the following substrates				Nu 683 concentration ($\times 10^{-8}$ M)	Percentage inhibition of the activity towards the following substrates			
	ACh	PrCh	BuCh	BCh		ACh	PrCh	BuCh	BCh
0·8	15	—	—	10	0·8	20	—	—	25
1·3	26	22	25	19	1·3	32	33	38	35
2·0	36	36	42	37	2·0	44	48	45	46
3·0	53	56	53	54	3·0	61	59	58	59
5·0	71	72	75	69	5·0	73	—	—	70
					8·0	86	82	85	85

for 30 min. (cf. Myers & Mendel, 1949). No summation of the activities towards acetylcholine and butyrylcholine was observed. Fractional precipitation experiments were carried out with the technique described to separate successive fractions at 0·29, 0·40, 0·51, 0·65 and complete (1·0) saturation with ammonium sulphate. About 70% of the total pseudo cholinesterase activity was recovered from the fraction precipitated between 0·51 and 0·65 saturation with ammonium sulphate but the specificity pattern of the pseudo cholinesterase was not altered, despite the fact that nearly all of the small amount of true cholinesterase activity present in the original serum was removed. This evidence strongly suggests that the hydrolysis of all three choline esters is due to a single enzyme.

It might be noted that the pseudo cholinesterase of horse serum hydrolyses caproylcholine more slowly than valeryl- or heptoyl-choline (Glick, 1941), and that the pseudo cholinesterase of human serum hydrolyses *iso*amyl valerate more slowly than *iso*amyl butyrate or caproate (Adams & Whittaker, 1949*a*). In view of these unexplained peculiarities, the specificity pattern of the pig serum enzyme should not exclude its classification as a pseudo cholinesterase.

The hydrolysis of acetyl-β-methylcholine by avian pseudo cholinesterases

The results obtained by Mendel *et al.* (1943) indicate that highly purified preparations of the pseudo cholinesterases from dog pancreas and horse serum hydrolyse acetyl-β-methylcholine at slightly less than 1% of the rate at which they hydrolyse acetylcholine, while Adams & Whittaker (1949*a*) measured a corresponding value of 1·4% for the pseudo cholinesterase of human serum. All of the mammalian preparations which appear in Fig. 1 hydrolysed acetyl-β-methylcholine at 3% or less of the rate of hydrolysis of acetylcholine. It is probable that most of these preparations were still contaminated with a small amount of true cholinesterase; these contaminations would have very little influence on the specificity patterns given in Fig. 1, since it was found that in all cases a true cholinesterase preparation (washed erythrocytes or brain) from the same mammalian species hydrolysed 0·045 M-acetylcholine a little less rapidly than it hydrolysed 0·03 M-acetyl-β-methylcholine.

Chicken serum contains an enzyme which rapidly hydrolyses acetyl-β-methylcholine. Mendel *et al.* (1943) showed that chicken serum exhibits a much higher activity towards acetyl-β-methylcholine than towards benzoylcholine, but hydrolyses acetylcholine most rapidly at high concentration. Earl & Thompson (1952), using butyrylcholine as a specific substrate for pseudo cholinesterase, reported that the hydrolysis of acetyl-β-methylcholine and butyrylcholine was catalysed by the same enzyme in chicken serum. This report has been confirmed. Fractional precipitation with ammonium sulphate did not give any evidence for the presence of a considerable amount of a true cholinesterase in the serum. The activities towards acetylcholine, butyrylcholine and benzoylcholine were all inhibited to the same extent by various concentrations of Nu 683 and of DFP (cf. Earl & Thompson, 1952), indicating that the same enzyme was responsible for the hydrolysis of all three esters. The Michaelis constant K_s with acetylcholine as substrate had an unexpectedly low value (Table 1 and Fig. 2). However, this enzyme is capable of hydrolysing tributyrin (Table 3) and exhibited maximum activity towards high concentrations of the aliphatic choline esters (Fig. 2).

These results, combined with the sensitivity of the enzyme to inhibition by Nu 683, indicate that the enzyme concerned could be classified as a pseudo cholinesterase. To support this conclusion, a comparison was made between the characteristics of the cholinesterase of chicken brain and that of chicken serum. The results obtained (Fig. 2) show

that the cholinesterases found in the serum and in brain are different enzymes. The true cholinesterase of brain, in contrast to the pseudo cholinesterase of serum, exhibits the phenomenon of excess substrate inhibition with acetylcholine (Fig. 2) and does not appreciably hydrolyse either tributyrin or butyrylcholine (Table 3). Both enzymes hydrolyse acetyl-β-methylcholine, but the shape and position of the activity-substrate concentration curves are different. Both enzymes hydrolyse only the same half, presumably the (+)-form, of the racemic substrate acetyl-(±)-β-methylcholine (cf. Glick, 1938).

rat-brain homogenate are also included in Fig. 2. A possible explanation of the asymmetry in these curves has been suggested elsewhere (Myers, 1952*b*).

Despite the high activity of the true cholinesterase towards propionylcholine, this enzyme failed to act appreciably on butyrylcholine. A crude homogenate of chicken brain hydrolyses butyrylcholine slowly, but this activity must be due almost entirely to the pseudo cholinesterase present (cf. Ord & Thompson, 1952; Earl & Thompson, 1952) since it could be inhibited selectively by the addition

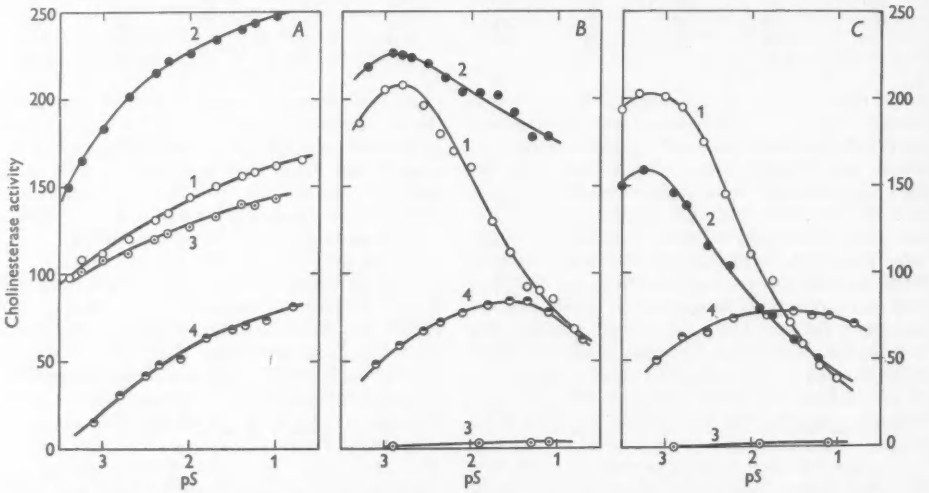

Fig. 2. Hydrolysis of ACh, PrCh, BuCh and MCh by (*A*) the pseudo cholinesterase of chicken serum, (*B*) the true cholinesterase of chicken brain, (*C*) the true cholinesterase of rat brain. The rates are plotted against −log (substrate concentration) (p*S*). ○—○, ACh; ●—●, PrCh; ⊙—⊙, BuCh; ◓—◓, MCh. The concentration of MCh is expressed as g.mol. acetyl-(±)-β-methylcholine/l. Rates are expressed as follows: pseudo cholinesterase of chicken serum as μl. CO_2/0·2 ml. solution/20 min.; true cholinesterase of chicken brain (measured in the presence of 4×10^{-9} M-DFP to inhibit any pseudo cholinesterase present) μl. CO_2/50 mg. fresh wt./20 min.; true cholinesterase of rat brain (measured in the presence of 10^{-8} M-DFP) μl. CO_2/75 mg. fresh wt./20 min.

Both the true cholinesterase and the pseudo cholinesterase of the chicken exhibit their maximal activity towards propionylcholine (Fig. 2). Certain of the true cholinesterases are known to exhibit approximately equal activities towards acetylcholine and propionylcholine (Augustinsson, 1949), but this appears to be the first example of a true cholinesterase which hydrolyses propionylcholine more rapidly than acetylcholine at optimal substrate concentrations. It is possible that this fact may be connected with the highly asymmetrical bell-shaped curves which are obtained on plotting the true cholinesterase activity against the logarithm of the substrate concentration (Fig. 2). For comparative purposes, the corresponding curves determined under similar experimental conditions with

of 4×10^{-9} M-DFP to the medium. The residual activity towards butyrylcholine in the presence of DFP was about 1% of the true cholinesterase activity towards acetylcholine (Table 3). Butyrylcholine is therefore a suitable specific substrate for determining the pseudo cholinesterase activity in the tissues of the chicken.

The pseudo cholinesterase preparations obtained from duck and pigeon sera also hydrolyse acetyl-β-methylcholine considerably more rapidly than the pseudo cholinesterases from mammalian sera (Table 3). This suggests that avian pseudo cholinesterases generally may exhibit appreciable activity towards this ester, although the pseudo cholinesterases of duck and pigeon hydrolyse acetyl-β-methylcholine much less rapidly than does the

chicken pseudo cholinesterase. The other substrate specificity characteristics of all of these pseudo cholinesterases are different from those of the true cholinesterases (Table 3).

Table 3. *Comparison of the rates of hydrolysis of MCh, tributyrin (TB) and BuCh by five pseudo cholinesterases and two true cholinesterases*

(The pseudo cholinesterase preparations were obtained from serum as described in the text. The true cholinesterase activities were determined with brain homogenate in the presence of a low concentration of DFP to inhibit any pseudo cholinesterase activity as described for Fig. 2. The activities towards MCh, TB and BuCh are expressed as a percentage of the cholinesterase activity towards optimal concentrations of acetylcholine, i.e. 0·045 M-ACh for the pseudo cholinesterases and approximately 0·001 M-ACh for the true cholinesterases (cf. Fig. 2). Since the hydrolysis of TB by the brain homogenates was not appreciably influenced by the addition of 10^{-6} M-eserine, the true cholinesterase activity towards TB must be less than 2% of the activity towards optimal concentrations of acetylcholine. These values and others given in brackets are approximations only since the activities are too low to allow accurate determinations.)

	Activity ratios		
Enzyme	$\frac{\text{MCh}}{\text{ACh}} \times 100$ (%)	$\frac{\text{TB}}{\text{ACh}} \times 100$ (%)	$\frac{\text{BuCh}}{\text{ACh}} \times 100$ (%)
Pseudo cholinesterase of			
Man	(1·2)	42	203
Pig	(0·7)	12	128
Duck	8	44	160
Pigeon	4	68	129
Chicken	42	58	89
True cholinesterase of			
Rat	37	(<2)	(0·5)
Chicken	42	(<2)	(1·0)

Specificity pattern of pseudo cholinesterases from different tissues of the rat

Various data have been published which indicate a variation in the specificity pattern of the pseudo cholinesterase from one tissue to another within the same animal as well as from one species to another. Calculations based on the data of Ord & Thompson (1950) show that the pseudo cholinesterases from intestinal mucosa, intestinal muscle and Harderian gland of the rat hydrolyse benzoylcholine at about 50% of the rate at which they hydrolyse acetylcholine, whereas the pseudo cholinesterases of rat heart and skin hydrolyse benzoylcholine at about 25% of the rate of acetylcholine. Similar variations were found by Sawyer & Everett (1947), although their data indicate that the pseudo cholinesterase of rat Harderian gland hydrolyses benzoylcholine at about 25% of the rate at which it hydrolyses acetylcholine. On the other hand, Goldstein (1951) found that the same type of pseudo cholinesterase is present in the serum, liver and gastric mucosa of the cat.

An attempt was made to confirm the results of Ord & Thompson (1950, 1951) by investigating the substrate specificity patterns of the pseudo cholinesterases from a series of rat tissues. To our surprise, the results did not give any indication of significant differences in the specificity pattern of the pseudo cholinesterases from different tissues of the rat. The results on comparative rates of hydrolysis of the various choline esters by rat heart ventricle agreed extremely well with those of Ord & Thomson (1951), and with those obtained on rat serum in the present investigation (Fig. 1). As judged by the close similarity of the specificity patterns obtained, the pseudo cholinesterases found in rat serum, heart ventricle, skin, pancreas, intestinal mucosa, intestinal muscle, uterus, multilocular adipose tissue (i.e. 'brown fat' or 'hibernating gland'), Harderian gland, parotid gland, lacrimal gland and mammary gland are all very similar if not identical. More limited investigations have been carried out on different tissues of the same animal in other species. The specificity patterns of the respective serum pseudo cholinesterases were found to be closely similar to those of the heart, Harderian gland and uterus of the hamster; the pancreas, heart and intestine of the ferret; the pancreas of the dog; the heart and liver of the mouse; and the liver and pancreas of the chicken. These results, in combination with those of Goldstein (1951), strongly suggest that the specificity pattern of the particular pseudo cholinesterase concerned is species specific but not organ specific.

Deviations from the usual pattern were observed with crude preparations of Harderian gland from the rat and hamster; in both cases it was found that the discrepancy was due to the presence of eserine-insensitive esterases capable of hydrolysing butyrylcholine but with very little activity towards either acetylcholine or benzoylcholine. Similar enzymes may be present in small amounts in the pancreas of the rat and intestine of the hamster.

Typical results obtained with a crude preparation of rat Harderian gland are given in Table 4; a portion of the total activity towards butyrylcholine is quite resistant to inhibition by any concentration of Nu 683 up to 10^{-3} M, whereas the pseudo cholinesterase activity is almost completely inhibited by 10^{-7}–10^{-6} M-Nu 683. The same portion of the activity towards butyrylcholine is not inhibited by a concentration (10^{-6} M) of eserine sufficient to inhibit the pseudo cholinesterase by about 90%. On the other hand, this esterase, like the pseudo cholinesterases, was sensitive to inhibition by low concentrations of DFP (10^{-7} M). Thus it appears to be similar to the enzymes found in the rabbit by

Table 4. *An esterase in rat Harderian gland which is capable of hydrolysing butyrylcholine but more resistant than the pseudo cholinesterase to inhibition by eserine and Nu* 683

(Harderian gland was obtained from adult female rats and homogenized with 9 vol. of water; the true cholinesterase activity towards 0·045 M-ACh was less than 2% of the total esterase activity. Native serum obtained from the same animals was also tested in the same way to allow comparison with an enzyme preparation containing only true cholinesterase and pseudo cholinesterase; the true cholinesterase was responsible for 6% of the total activity of the serum towards 0·045 M-ACh. The enzyme preparations were incubated at 37·5° for 30 min. with Nu 683 or eserine in the usual manner before measuring the residual esterase activity.)

	Nu 683 as inhibitor						Eserine as inhibitor	
	Esterase activity of serum towards the following substrates		Esterase activity of Harderian gland towards the following substrates				Esterase activity of Harderian gland towards the following substrates	
Concentration of inhibitor (M)	ACh (μl. CO_2/0·2 ml./20 min.)	BuCh	ACh	PrCh (μl. CO_2/20 mg. fresh wt./20 min.)	BuCh	BCh	ACh (μl. CO_2/20 mg. fresh wt./20 min.)	BuCh
0	121	156	106	266	171	24·8	107	175
10^{-8}	45·0	58·2	43	117	87	9·5	—	—
10^{-7}	10·5	12·5	14·6	47·9	48·2	2·5	61	135
10^{-6}	2·5	2·0	3·9	30·1	37·6	0·0	11·2	57·9
10^{-5}	0·0	0·0	2·0	28·0	36·2	0·0	3·1	38·5
10^{-4}	0·0	0·0	1·8	27·7	36·9	0·0	1·2	4·9
10^{-3}	—	—	—	—	32·6	—	—	—

Table 5. *Inhibition of the cholinesterases in the blood of mice injected with DFP*

(Female mice weighing 22–25 g. were injected intraperitoneally with solutions of DFP in saline; after observing the mice for 1 hr. subsequently to the injection, the animals were sacrificed and pooled samples of oxalated blood were collected from each group. The cholinesterase activities were determined in the usual manner using 0·03 M-acetyl-β-methylcholine as specific substrate for the true cholinesterase and 0·006 M-benzoylcholine for the pseudo cholinesterase.)

		True cholinesterase		Pseudo cholinesterase		
No. of mice	Amount of DFP injected (μg.)	Activity (μl. CO_2/ml. blood/20 min.)	Inhibition (%)	Activity (μl. CO_2/ml. blood/20 min.)	Inhibition (%)	Remarks
15	0	97·6	—	170·0	—	—
4	4	105·3	0	28·8	83	No symptoms
12	8	82·5	15	5·0	97	No symptoms
4	20	47·5	51	0·0	100	No symptoms
4	40	22·7	77	0·0	100	Listless
8	100	8·8	91	0·0	100	Severe fibrillation and diarrhoea

Koelle (1952); in view of their low activity towards benzoylcholine, these eserine-resistant esterases could hardly be designated as 'benzoyl-cholinesterases' (cf. Sawyer, 1945). Further studies on the substrate specificity and occurrence of these enzymes are being carried out; to avoid confusion with the true cholinesterases and pseudo cholinesterases, they will be referred to in the present publication simply as eserine-resistant enzymes which can hydrolyse butyrylcholine (cf. Koelle, 1952).

Inhibition of the pseudo cholinesterase in mice

The results given in Table 1 and Fig. 1 seem to justify a classification of all of these enzymes as pseudo cholinesterases, even though their specificity patterns show considerable variation from species to species. To substantiate the physiological classification of these enzymes as pseudo cholinesterases, it was demonstrated previously that selective inhibition of this enzyme in dog and man by Nu 683 (Hawkins & Gunter, 1946) and DFP (Hawkins & Mendel, 1947; cf. Mazur & Bodansky, 1946), respectively, does not cause any observable symptoms of acetylcholine poisoning. The pseudo cholinesterases both of dog and of man are, however, butyro-cholinesterases; since the enzyme found in mouse serum exhibits a quite different type of specificity pattern (Fig. 1), it was necessary to show that an enzyme such as this is also a pseudo cholinesterase physiologically, i.e. that it is not essential for the hydrolysis of the neurohumoral choline esters *in vivo*. Several groups of female mice were therefore injected with graded doses of DFP in aqueous solution; after observing the mice for 1 hr. subsequently, the animals were sacrificed and the

cholinesterase activities determined in pooled samples of the blood. The results of this experiment (Table 5) are almost identical with those obtained on dogs by Hawkins & Gunter (1946). No signs are observed when the pseudo cholinesterase activity is completely inhibited by a selective inhibitor. Signs of acetylcholine accumulation appear only when the true cholinesterase activity is inhibited more than 75–80%.

The ali-esterase (tributyrinase) of mouse serum proved to be even more sensitive than the pseudo cholinesterase to inhibition by DFP *in vivo*. This enzyme therefore appears to resemble the tributyrinase of rat serum, which behaves similarly *in vitro* (Myers, 1952*c*).

DISCUSSION

The physiological distinction between true cholinesterase and pseudo cholinesterase

Although the two types of cholinesterases were first distinguished by differences in enzymological characteristics, the original classification according to physiological function (Mendel & Rudney, 1943) has been supported by the results of subsequent investigations. The pseudo cholinesterase in the intact animal can be completely inhibited without causing any signs of acetylcholine accumulation (Hawkins & Gunter, 1946; Hawkins & Mendel, 1947; cf. Mazur & Bodansky, 1946). On the other hand, inhibition of the true cholinesterases by more than 70–80% leads to acute acetylcholine poisoning, while complete inhibition is invariably fatal (Gunter & Mendel, 1945; Hawkins & Gunter, 1946; Hawkins & Mendel, 1947, 1949; Nachmansohn & Feld, 1947). Experiments with various isolated organs usually show a close correlation between the inhibition of true cholinesterase and the pharmacological effects of the cholinesterase inhibitors (Blaschko, Bülbring & Chou, 1949; Kordik, Bülbring, & Burn, 1952; Kamijo & Koelle, 1952; cf. Koelle, Koelle & Friedenwald, 1950).

While no animal species devoid of true cholinesterase has been found, the sera and tissues of sheep and ox do not contain measurable amounts of pseudo cholinesterase (Mendel *et al.* 1943; Gunter, 1946), although they may contain eserine-resistant enzymes capable of hydrolysing choline esters. This has been confirmed by other workers; ox heart ventricle (Nachmansohn & Rothenberg, 1945) and adrenal gland (Langemann, 1951) show no significant activity towards benzoylcholine, in contrast to the heart (Ord & Thompson, 1950) and adrenal gland (Sawyer & Everett, 1947) of the rat.

The physiological function of the pseudo cholinesterases is still uncertain. Chance observations made in the present investigation suggest that it is unwise to draw any general conclusions regarding the function of the pseudo cholinesterases from a study of its distribution in different tissues of a given animal. For example, the pseudo cholinesterase activity of rat heart is very high (Ord & Thompson, 1950, 1951), but that of hamster heart was relatively low and there was almost no pseudo cholinesterase activity to be found in squirrel heart. A parallel observation is found in the results of Mendel *et al.* (1943), which show that the erythrocytes of avian species contain very little true cholinesterase activity despite the fact that mammalian erythrocytes often exhibit a high activity.

Recent results suggest that propionylcholine, as well as acetylcholine, might play a role as a neurohumoral transmitter in some cases (Banister, Whittaker & Wijesundra, 1951), but this would not alter the significance of the experimental observations on the pharmacological effects of the inhibition of true cholinesterase and pseudo cholinesterase. Whatever the physiological function of the pseudo cholinesterases may prove to be, the original distinction between them and true cholinesterases does seem to be justified by the primary physiological importance of the true cholinesterases for the rapid hydrolysis of acetylcholine in the intact animal.

Enzymological classification of the cholinesterases

A strict enzymological classification of the cholinesterases on the basis of their specificity patterns will often be confusing because the specificity pattern of the same type of enzyme varies from one species to another. The use of terms such as butyro-cholinesterase and propiono-cholinesterase may be useful to characterize the specificity pattern of the particular pseudo cholinesterase. However, the enzymological properties of an enzyme are typical of the species and vary within an apparently continuous range of specificity pattern; a particular enzyme must therefore be designated according to its source. It is also interesting that the pseudo cholinesterase of the pig does not show a marked preference for any one particular acyl group; the significance of this is not understood but it is obvious that such an enzyme would be difficult to classify on an enzymological basis.

A further complication is presented by the fact that the rate of enzymic hydrolysis of an ester will depend in part upon the physico-chemical properties of the ester, for example, the solubility, the surface activity and the inherent stability of the ester. For example, there may be a correlation between the rapid enzymic hydrolysis and the high rate of spontaneous hydrolysis of the haloacetate esters (cf. Adams & Whittaker, 1950). Thus a portion of what has been called specificity of an esterase may not be enzyme specificity at all but a function of the

lability of the bond that is exposed to the action of the enzyme (Smith, 1951).

A nomenclature based on specificity patterns is also disadvantageous when more than one type of enzyme exhibits similar specificity characteristics. In particular, this terminology fails to stress the characteristic differences between true cholinesterases, pseudo cholinesterases, and eserine-resistant enzymes which hydrolyse butyrylcholine. The limited data available on these eserine-resistant esterases indicate that some of these enzymes may also be butyro-cholinesterases, and the data given above (Fig. 1) point to the possibility that a pseudo cholinesterase with maximal activity towards acetylcholine may be found in species which have not yet been investigated. One example of a true cholinesterase which hydrolyses propionylcholine more rapidly than acetylcholine has been found (Fig. 2) and the true cholinesterases from certain invertebrates exhibit a greater activity towards acetyl-β-methylcholine than towards acetylcholine (Augustinsson, 1946; Metcalf & March, 1950).

Despite the fact that it is becoming more and more apparent that there are many types of cholinesterases from an enzymological viewpoint, it does seem justifiable and useful to retain the distinction between the two main groups of eserine-sensitive cholinesterases in vertebrate physiology. Apparent exceptions to this general classification can usually be explained on the basis of species variations in specificity patterns; the results with the pseudo cholinesterases of pig serum (Levine & Suran 1950), rat heart ventricle (Ord & Thompson, 1951) and chicken serum (Earl & Thompson, 1952) are of especial interest in this connexion and emphasize the importance of designating the particular pseudo cholinesterase or true cholinesterase according to the species from which it was obtained. The particular nomenclature which is used by various authors to distinguish these two groups of eserine-sensitive cholinesterases in mammalian tissues is not of great importance; however, the designation should not leave any room for ambiguity, and we believe that the evidence cited above still justifies a terminology which indicates a distinction according to physiological importance, e.g. true cholinesterase and pseudo cholinesterase.

The differentiation of true cholinesterase and pseudo cholinesterase activity

The main characteristics of a pseudo cholinesterase, as originally put forward by Mendel and co-workers, were that the enzyme should hydrolyse acetylcholine, benzoylcholine, tributyrin and methyl butyrate but not acetyl-β-methylcholine, and that the activity towards any ester should be inhibited by low concentrations of eserine. The true cholinesterases, on the other hand, should hydrolyse acetylcholine and acetyl-β-methylcholine but not benzoylcholine, tributyrin or methyl butyrate. Unlike the pseudo cholinesterases, the true cholinesterases exhibit maximal activity towards acetylcholine at low concentrations of this ester and are inhibited by excess of acetylcholine; like the pseudo cholinesterases, the true cholinesterases are inhibited by low concentrations of eserine. Tributyrin and methyl butyrate are also hydrolysed by the aliesterase, but these latter enzymes do not hydrolyse choline esters and are not inhibited by low concentrations of eserine.

The use of specific substrates. This was proposed by Mendel *et al.* (1943) and still appears to be the most convenient method for identification and quantitative measurement of the true cholinesterase and pseudo cholinesterase activity in various mammalian tissues (cf. Sawyer & Everett, 1947; Ord & Thompson, 1950), but it should always be supplemented by a test for sensitivity to inhibition by low concentrations of eserine and prostigmine or prostigmine analogues. Both benzoylcholine (Mendel *et al.* 1943) and butyrylcholine (Cohen *et al.* 1949; cf. Nachmansohn & Rothenberg, 1945) have been proposed as specific substrates for the measurement of pseudo cholinesterase activity, but other esterases which can hydrolyse one or both of these esters are known to exist (Sawyer, 1945; Koelle, 1952) and may be more widespread in occurrence than previously considered; these enzymes, unlike the true cholinesterases and pseudo cholinesterases, are not inhibited by 2×10^{-6}M-eserine (cf. Gunter, 1946; Hawkins & Gunter, 1946).

Since the specificity patterns of the pseudo cholinesterases differ greatly, the measured activity towards either butyrylcholine or benzoylcholine does not constitute an exact basis for comparison of the 'amount' of pseudo cholinesterase in the serum of different mammalian species, nor does it give an indication of the relative activity towards other esters. However, if the specificity pattern is constant from tissue to tissue within the same animal, we are justified in comparing the pseudo cholinesterase activities of these different tissues towards one of the specific substrates.

As pointed out by Adams & Whittaker (1949*a*), it may be unwise to attach too much significance to very small rates of hydrolysis of these substrates by enzyme preparations or to attempt to measure very low concentrations of the enzyme for which they are specific. The very small activity of the mammalian pseudo cholinesterases towards acetyl-β-methylcholine does not interfere greatly with the use of this ester as a specific substrate for determination of the true cholinesterase activities of mammalian tissues, but a complication is introduced by the fact that the pseudo cholinesterase of chicken serum also

hydrolyses acetyl-β-methylcholine (Earl & Thompson, 1952). Thus it is not possible to use this ester as a specific substrate for measurement for true cholinesterase activity in the chicken, and the same difficulty may be encountered in other non-mammalian species. However, since butyrylcholine is suitable as a specific substrate for the determination of pseudo cholinesterase activity in the chicken, an estimation can be made of the residual fraction of the total cholinesterase activity towards acetylcholine which must be due to true cholinesterase present.

The phenomenon of excess substrate inhibition. Excess substrate inhibition seems to characterize the activity of the true cholinesterases towards acetylcholine, whereas the pseudo cholinesterases do not show it. The concentration of acetylcholine at which inhibition may be observed varies considerably with the true cholinesterases from lower forms of life (Hawkins & Mendel, 1946; Augustinsson, 1946, 1948, 1949) and one true cholinesterase is known (in *Planaria*) which exhibits its maximal activity towards relatively high concentrations of acetylcholine (Hawkins & Mendel, 1946). Moreover, this characteristic, like the use of specificity patterns, is of very limited value for assaying mixtures of true cholinesterase and pseudo cholinesterase.

The ability of a cholinesterase to hydrolyse tributyrin. This can generally be used as additional evidence for its classification as a pseudo cholinesterase, but the lack of an appreciable eserine-sensitive activity towards tributyrin does not allow any conclusion as to the type of cholinesterase concerned. The pseudo cholinesterases of man, horse and dog all hydrolyse tributyrin and methylbutyrate at an appreciable rate (Mendel & Rudney, 1943; Adams & Whittaker, 1949*b*), while the true cholinesterases of man, horse, dog, sheep, ox and mouse show no significant activity towards these esters (Mendel & Rudney, 1943). However, the pseudo cholinesterase of rat heart is characterized by the absence of an appreciable activity towards tributyrin (Wright, 1946; Ord & Thompson, 1951).

The pseudo cholinesterases of all the rat tissues studied in the present investigation proved to have the same specificity characteristics as that of rat heart. Thus it is not surprising to find recorded in an early paper that the cholinesterase of rat skin does not hydrolyse tributyrin (Thompson & Whittaker, 1944), even though the major portion of the acetylcholine hydrolysis by rat skin is due to a pseudo cholinesterase (Ord & Thompson, 1950). It is also of interest that a cholinesterase preparation purified from mouse liver (Mendel & Mundell, 1939, unpublished) also failed to act appreciably on tributyrin; since this cholinesterase preparation hydrolysed acetylcholine most rapidly at high concentrations of this ester and was inhibited by low concentrations of eserine, we can conclude that this enzyme must be another example of a pseudo cholinesterase which does not hydrolyse tributyrin.

Selective inhibitors. Selective inhibitors have been used by many workers for differentiating the activities of various esterases. Under the experimental conditions used by Mendel and co-workers (cf. Mendel & Hawkins, 1950), a concentration of 10^{-7}–10^{-6}M-eserine is usually sufficient to inhibit the true cholinesterase and pseudo cholinesterase activities of mammalian tissues by 90–100%. The ali-esterases, on the other hand, are inhibited only by much higher concentrations of eserine (approx. 10^{-3}M). The Harderian gland of the rat contains an enzyme which hydrolyses butyrylcholine and which is not appreciably inhibited by 10^{-6}M-eserine but is almost completely inhibited by 10^{-4} M-eserine; thus there is not a very large margin between the concentrations of eserine necessary to inhibit completely the cholinesterases of mammalian tissues and the concentrations which partially inhibit other enzymes, but this margin is probably sufficient to serve as an experimental criterion for identifying the cholinesterase activities of mammalian tissues (cf. Gunter, 1946; Myers & Mendel, 1949). However, the true cholinesterases found in lower forms of life may be considerably less sensitive to inhibition by eserine than are the cholinesterases of mammalian tissues (Hawkins & Mendel, 1946) so that caution must be used in interpreting these results. Prostigmine and analogues have not been as extensively investigated but these inhibitors might give slightly better results with the cholinesterases in lower forms of life (Hawkins & Mendel, 1946), and the data given above (Table 4) show that Nu 683 is much more suitable than eserine for distinguishing pseudo cholinesterase activity from that due to other enzymes which hydrolyse butyrylcholine.

Other investigators have used the sensitivity to inhibition by DFP as a criterion for distinguishing true cholinesterase and pseudo cholinesterase activity (Adams & Thompson, 1948; Ord & Thompson, 1950). Considerable information concerning the occurrence of true cholinesterase and pseudo cholinesterase in different tissues has been obtained by this means (Ord & Thompson, 1950, 1952), and the same technique proved to be extremely useful for the present investigation. However, it has been found that DFP and other alkyl phosphate derivatives may inhibit a great many types of hydrolytic enzymes including the eserine-resistant esterases which hydrolyse butyrylcholine (Koelle, 1952) and certain ali-esterases (Myers & Mendel, 1949; Myers, 1952*c*). The method using DFP to differentiate true cholinesterase and pseudo cholinesterase activities should be supplemented, therefore, by a test with

eserine and prostigmine or prostigmine analogues to show that the esterase activity in question is not due to other types of hydrolytic enzymes.

SUMMARY

1. The pseudo cholinesterases from the sera of ten mammalian and three avian species have been studied in detail. It was found that eight of these pseudo cholinesterases can be classified as butyrocholinesterases and four as propiono-cholinesterases, while the pseudo cholinesterase of pig serum could not be classified readily on the basis of its substrate specificity pattern alone. However, there is no clear-cut distinction between the two groups of pseudo cholinesterases; the substrate specificity patterns can be arranged into a series which shows a gradual, progressive shift in the optimal acyl group.

2. Closely similar specificity patterns were found for the pseudo cholinesterase in twelve different rat tissues, indicating that the enzyme is probably the same in all tissues. This conclusion is substantiated by the results of more limited investigations in other species; thus the pseudo cholinesterases appear to be species specific but not organ specific.

3. The Harderian glands of the rat and hamster also contain an eserine-resistant esterase capable of hydrolysing butyrylcholine.

4. In contrast to the true cholinesterases previously investigated, the true cholinesterase of chicken brain hydrolyses propionylcholine more rapidly than acetylcholine. It resembles other true cholinesterases in its remaining enzymological properties, which differ greatly from those of the pseudo cholinesterases; nevertheless, a rigid classification of the cholinesterases on the basis of substrate specificity patterns would characterize both the true cholinesterase and the pseudo cholinesterase of the chicken as propiono-cholinesterases.

5. Acetyl-β-methylcholine cannot be used as a specific substrate for measurement of true cholinesterase activity in the chicken; this seems to apply, to some extent, to avian species generally. However, butyrylcholine appears to be suitable as a specific substrate for measurement of the pseudo cholinesterase activity in these species (cf. Earl & Thompson, 1952).

6. The proposed classification of cholinesterases on the basis of substrate specificity patterns has been criticized, and the importance of designating the pseudo cholinesterases according to their source has been stressed.

The author is much indebted to Prof. B. Mendel for his continued interest in this investigation, and to Miss M. de Jonge and Miss E. Simons for invaluable technical assistance. The initial investigations were made possible through the courtesy of Prof. R. H. S. Thompson, London, who kindly supplied us with samples of propionyl- and butyryl-choline perchlorates, and Dr J. A. Cohen, Leiden, who supplied us with samples of the chlorides. Samples of acetyl-, propionyl- and butyryl-choline perchlorates were later synthesized by A. Kemp, jun., in this laboratory.

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An Apparatus for the Spectrokinetic Study of Rapid Reactions

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The kinetic study of any chemical reaction requires the creation of a homogeneous, reacting mixture accessible to analysis at any instant during the change. For rapid reactions, this technical problem was first solved in a direct and general manner by Hartridge & Roughton (1923). Their method was based on the constant flow principle: the reactants were driven at high speed through separate jets into a small chamber, where rapid mixing occurred. The reacting mixture flowed thence at constant rate down a tube of uniform bore, and could be analysed by optical or other physical means at any distance along the tube. Originally the analysis was made with a reversion spectroscope; later thermal and photoelectric methods were used (Roughton, 1930; Bateman & Roughton, 1935; Roughton & Millikan, 1936). The validity of the technique was established by various controls (Hartridge & Roughton, 1923; Roughton & Millikan, 1936).

Modifications of the method were devised by Millikan and by Chance, with rather different aims in view. In order to extend the work of Hartridge and Roughton on sheep haemoglobin to other oxygen-carrying pigments, Millikan (1936) adapted the constant-flow apparatus to the use of small quantities of material, by replacing the original gas-pressure drive with motor-driven syringes and using a sensitive differential photoelectric colorimeter to analyse the flowing mixture. The apparatus of Chance (1940, 1951) was designed for the kinetic study of enzyme reactions and embodied fundamental alterations in technique. The requisite extension of the time range and economy of material were secured with an accelerated flow method and a very sensitive reaction meter with a high speed of response: a manual syringe drive gave an initial impetus to the reactants, and the subsequent variations of flow velocity, and therefore of time interval, and the corresponding variations in the extent of reaction, measured photoelectrically at a fixed distance from the mixing chamber, were continuously recorded with a double-beam cathode-ray tube. The complete kinetic curve was obtained directly as a trace, which could be photographed.

A modification of the Hartridge-Roughton apparatus, constructed for kinetic studies of human haemoglobin with precision as the major objective of the design, is described in this paper. The apparatus consists essentially of a constant-flow apparatus of the gas-pressure type and a photoelectric spectrophotometer (Beckman quartz Model DU), and can be used to record the absorption spectra of transient intermediate compounds (Dalziel & O'Brien, 1951, 1952*a*, *b*).

1. DESIGN AND USE OF THE APPARATUS

Construction

General features. The apparatus is shown diagrammatically in Fig. 1. The reactants are stored at constant temperature in vessels surrounded by water in a lagged pressure chamber, and are forced through capillary jets into the observation tube, which can be moved back and forth in the light beam from the spectrophotometer. Slower reactions are followed in a water-jacketed extension to the observation tube, after the flow has been stopped.

Reactant vessels and propulsion. The four 1 l. glass reactant vessels, anchored by studs fixed on to the glass and bearing upwards on a heavy metal stand, are connected by glass siphon tubes and rubber junctions to outlets in the wall of the pressure chamber. The four outlets are glass tubes cemented into metal sleeves, and lead to two spring-loaded three-way glass stopcocks, and thence to the two jets. The stopcocks permit alternate flow of the reacting mixture and calibration fluids. The stopcock barrels are 1 in. diameter by $1\frac{1}{2}$ in. length, and if carefully made and greased with Apiezon L will contain pressures of up to 50 lb./sq.in. They are clamped to a vertical plate screwed to the angle piece

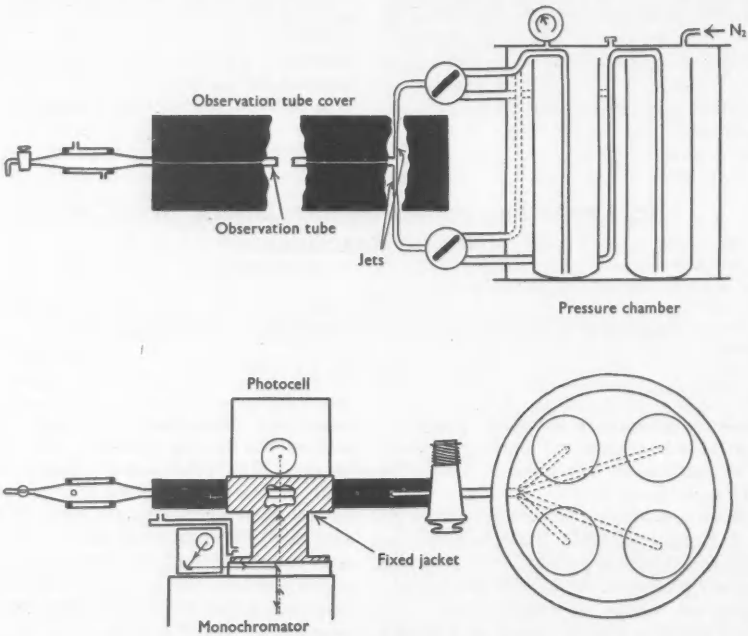
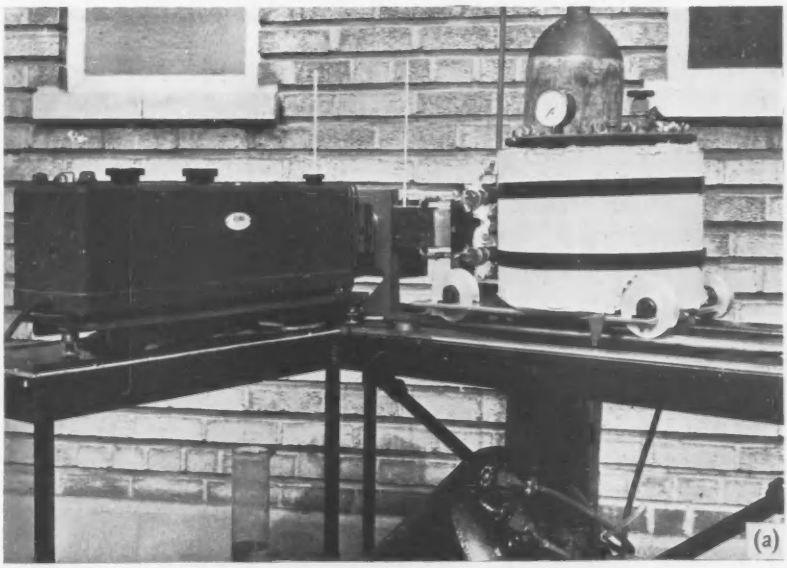


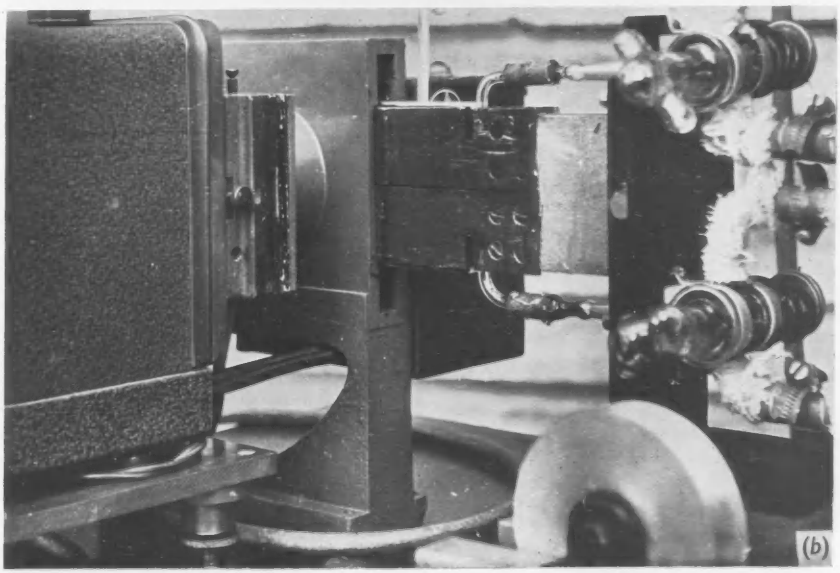
Fig. 1. Diagram of apparatus (not to scale).

Framework. The apparatus must be rigid, and the movement of the observation tube in the light path smooth and reproducible. The entire constant-flow apparatus is permanently mounted on a substage which travels along one limb of an L-shaped table, and the spectrophotometer is put in place on the other limb (Pl. 1*a*). A rectangular block containing the observation tube moves through a fixed metal jacket which replaces the cell compartment of the spectrophotometer, and about which the spectrophotometer may be assembled when required. The distance between two points of observation is read off on a scale suitably placed to indicate the distance travelled by the substage. The table top of $\frac{1}{2}$ in. bakelite is screwed to a steel frame supported by metal tubing. The substage, $\frac{3}{8}$ in. steel plate, runs on four wheels along two $\frac{3}{4}$ in. diameter steel rails screwed to the table, and the movement is controlled by a screw drive.

which holds the jet-observation tube unit. The rubber connexions are either canvas reinforced or pressure tubing with clamps, and are wired on and sealed to the glass with Bostik C. The pressure tank is $\frac{3}{8}$ in. welded steel, 10 in. diam. by 10 in. high, tested hydraulically to 120 lb./sq.in. Pressure is applied from a nitrogen cylinder through a reducing valve and a 60 l. tank which acts as a pressure stabilizer.

Jets, mixing chamber and observation tube. These parts (Fig. 2) are assembled as a single unit enclosed in a flat rectangular metal block (the observation tube cover) with a longitudinal slit through which measurements may be made right up to the mixing chamber without interference by external light. This arrangement is facilitated by a simple jet-mixing chamber unit, made by drilling into the wall of a glass capillary tube, first with a fine drill until the tip just enters the capillary, and then with a cylindrical grinding





The rapid reaction apparatus. (*a*) Complete assembly. (*b*) Detail of spectrophotometer assembly.

tool. Thus, two opposed jets open into the end of a cylindrical mixing chamber 2–3 mm. long. The observation tube, a 30 cm. length of Veridia precision-bore glass tubing (Chance Bros. Ltd.) ground at the end to fit the curved surface of the jet unit, is cemented over the mixing chamber with Araldite (Aero Research Ltd.). To interchange jets and observation tubes of various dimensions, this joint can be severed by burning the cement. For studies of the deoxygenation of haemoglobin and the dehydration of carbonic acid, reactions with half periods of about 0·05 sec., the following dimensions proved convenient: jets 2·5 cm. long and 0·5–1 mm. diameter, mixing chamber 1–2 mm. diameter, and observation tube 2 mm. diameter.

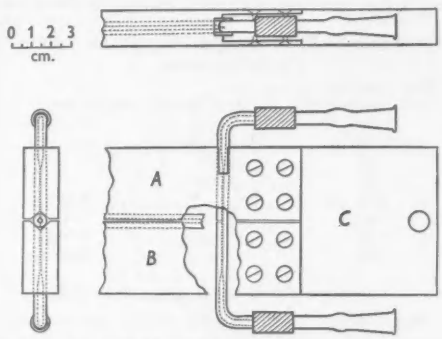


Fig. 2. Jets, observation tube and observation tube cover assembled.

The observation tube cover consists of three blocks of dural, painted flat black to reduce reflexion. To ensure that all light reaching the photocell passes through the reaction mixture, the observation tube is held rigidly between two of the blocks, *A* and *B*, in V-shaped grooves, accurately milled to leave a uniform aperture of 1 mm. along the length of the cover, inside which the 2 mm. bore tube is aligned with its axis along the centre of the aperture. The third block, *C*, completes the enclosure of the jets, and is clamped in a slot in a heavy angle piece screwed to the substage. The jets are connected to the stopcocks by canvas-rubber junctions and ground-glass joints sealed with Chadwick's wax, which permit detachment of the complete jet-observation tube unit from the rest of the constant-flow apparatus.

Spectrophotometer assembly. The observation tube cover travels through a close-fitting brass jacket, fixed to the table, with clearance for the jet leads to permit observations up to the mixing chamber (Pl. 1*b*). The Beckman monochromator and photocell box are assembled around the jacket by means of screws and alignment pins, and the dimensions are such as to allow travel of the observation tube past the lamp house and its water cooler (Fig. 1). An adjustable vertical slit inside the jacket defines the length of the observation tube illuminated.

Methods of operation

It will be assumed that only one of the two reactants, reactant *R*, is coloured, in which case the absorption spectrum of the reaction mixture at the instant of mixing will be that of reactant *R* appropriately diluted with solvent. To record the changes of spectral absorption accompanying the reaction, two of the reactant vessels are filled with the reactants and the other two with solvent, so arranged that each jet will deliver either a reactant or solvent. The extinction of the reaction mixture, E_M, for light of a particular wavelength is determined at intervals during the reaction by measurements on the flowing mixture at various distances from the mixing chamber. Small differences between the optical depth at the several points of observation are corrected for by parallel measurements on *R* mixed with solvent. The procedure at each point of observation is: (1) with both jets delivering solvent, the spectrophotometer is set to $E=0$ in the usual way, (2) reactant *R* and solvent are run together, and the extinction $E_R=E_{M_0}$ recorded, (3) with both reactants running together, E_{M_t} is measured, and finally (4) solvent is run from both jets and the zero checked. The time *t* is calculated from the distance of the point of observation from the mixing chamber, the cross-sectional area of the observation tube, and the measured volume flow rate. For measurements at times greater than 2 or 3 sec., the flow is stopped abruptly by closing the stopcock at the end of the observation tube, and the time course of the extinction is followed with a stop-watch.

The plot of E_{M_t} against *t* may be called a spectrokinetic curve for the reaction, and from the curves for a number of wavelengths the absorption spectrum of the reaction mixture can be built up at any stage of the reaction. Consecutive reactions may be indicated by simultaneous inflexions, maxima or minima in the spectrokinetic curves for several wavelengths, and the absorption spectrum of the mixture may permit characterization of the intermediate compounds (cf. Dalziel & O'Brien, 1952*a*).

If the reaction is a simple one, in which *R* is transformed directly into a single stable product *P* by an isolated or reversible reaction, a series of absorption curves will be obtained intersecting at the isosbestic points of *R* and *P*, and if Beer's law holds the spectrokinetic curve at an appropriate wavelength may be transposed into a concentration-time curve. Consideration of the relationships involved, however, shows that more accurate kinetic measurements can be obtained by means of calibration fluids corresponding to 0 and 100% reaction (Appendix). The wavelength should be chosen according to the concentration of the initial reactant to give an extinction change of about 0·8 for the complete reaction. The calibration fluids are put in the reactant vessels which previously contained solvent, and the procedure for each measurement is: (1) the calibration fluid having the smaller extinction coefficient is run through the observation tube from one jet, and the spectrophotometer set to

$E = 0$; (2) the other calibration fluid is run from the other jet, and the measured extinction is $\Delta E_{100\,\%}$, the extinction change for complete reaction; (3) the reactants are run together and the extinction ΔE_t recorded; and finally (4) the zero setting is checked by repeating (1). Assuming that the 100% calibration fluid has the smaller extinction, then the concentration of reactant R at time t, expressed as a fraction of the initial concentration, is $y = \Delta E_t / \Delta E_{100\,\%}$; the analysis is independent of the optical depth and of errors of the wavelength scale.

The calibration fluids are prepared beforehand by mixing reactant R with solvent and with the second reactant. The correct proportions, which depend upon the relative delivery rates of the two jets, can be obtained by mixing the solutions in the apparatus, but transient fluctuations of the relative delivery rates, due to the passage of debris or stopcock grease, occasionally occur. The jets deliver at almost equal rates, and it is better to prepare the calibration fluids on this assumption, and to collect two or three samples of the reaction products from the apparatus during the experiment for comparison with the 100% calibration fluid in optical cells. Correction can then be made for small differences of concentration between the calibration fluids and the reaction mixture, and the initial reactant concentrations can be calculated.

Validity and accuracy of the method

The performance of the component parts of the apparatus, and the factors upon which the validity and precision of the method depend will be considered individually.

Mixing tests

The efficiency of mixing was investigated by the thermal method of Hartridge & Roughton (1924), which consists in mixing strong acid and base in the apparatus and measuring the increase of temperature of the flowing mixture, which is a measure of the extent of mixing, at various distances along the tube.

Copper-constantan needle thermocouples (Roughton & Millikan, 1936) were used. The elements were enclosed in a steel tube, 0·7 mm. external diameter, and the hot junction at one end of the tube was insulated and protected with an acid- and alkali-resistant bakelite varnish. The cold junctions, dipping into mercury contacts, were kept at constant temperature in a lagged vacuum-walled flask containing water at about room temperature. With a Cambridge 'spot' galvanometer of 400Ω resistance a deflexion of 1 cm./° C. was obtained. The mixing tests were made with approximately 2N-NaOH and 2N-H_2SO_4, the overall temperature increase being 13°. The concentrations were adjusted, according to the relative flow rates through the jets, to give exact neutralization in the apparatus, a condition essential to the validity of the method.

Results obtained with two-jet capillary mixing units of the type described earlier are summarized in Table 1. With jets of 0·5 and 1 mm. diameter, and mixing chambers of 1 and 2 mm. diameter, mixing occurs to the extent of 90% or more in the mixing chamber and reaches 99% at a distance of 0·5–1 cm. along the observation tube over a range of flow rates. The jets of smaller bore give the more rapid mixing: at a flow rate of about 140 cm./sec. down the observation tube, 99% mixing is achieved in 0·005 sec. with 0·5 mm. jets, and in 0·008 sec. with 1 mm. jets, whilst at the higher flow rate of 280 cm./sec., 1 mm. jets also give 99% mixing in 0·005 sec.

Table 1. *Efficiency of mixing with two-jet capillary units*

Flow velocity in 2 mm. observation tube (cm./sec.)	Time for 99% mixing (sec.)	
	0·5 mm. jets	1 mm. jets
60	0·017	—
85	0·008	—
110	—	0·012
140	0·005	0·008
210	—	0·006
280	—	0·005

Character of flow in the observation tube

The validity of the constant-flow method rests upon the assumption of an ideal type of flow in which all parts of the liquid move at the same rate down the observation tube. In practice, the velocity, and therefore the extent of reaction, will not be uniform over a cross-section. The observed extent of reaction at any distance along the tube will depend upon the radial velocity distribution and the way in which measurements are made; it will not be, in general, the true value corresponding to the mean time of arrival of the fluid at the cross-section, the time calculated from the measured volume flow rate.

Roughton & Millikan (1936) showed theoretically that, with turbulent flow in the observation tube, errors from this source would be negligible, but with streamline flow, the observed percentage reaction might be up to 7% in error. They showed experimentally, however, that for flow rates theoretically in the streamline region (down to one-sixth of the critical velocity) the actual radial velocity distribution in the observation tube was much more uniform than for streamline flow, and calculated that the error of measurements of the percentage reaction would not normally exceed 0·8%. These conclusions could not be assumed valid for the present apparatus, since the calculations involved an approximation justified only if the whole cross-section of the tube were illuminated and if the light absorption were very small (Millikan, 1932). The analysis has therefore been modified to cover the more general case.

If only part of the cross-section of the observation tube is illuminated, the observed extent of reaction must lie somewhere between the values corresponding to the light absorption along a diameter and along the extreme chord. Calculations have been made, for streamline and turbulent flow, of the mean percentage reaction along each of these paths, for several assumed values of the true percentage reaction.

Method. Consider a cross-section of the observation tube, Fig. 3, and let $\bar{t}$ be the average time of arrival of the fluid at this cross-section; this will be the measured time for the cross-section in the constant-flow method, calculated from the volume flow rate, the distance from the mixing chamber,

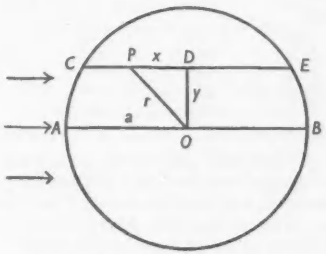


Fig. 3. Cross-section of the observation tube, for calculations on character of flow.

and the radius of the tube, a. Let P be any point in the cross-section distance r from the centre, and CDE a chord through P distance y from the centre and parallel to the light. Then: (i) for a given radial velocity distribution (assumed to be set up at the mixing chamber) the time of arrival of the fluid at P can be expressed in terms of $\bar{t}$ and r; (ii) for any assumed true extent of reaction in the measured time $\bar{t}$, the corresponding actual extent of reaction at P can be expressed as a function of r; and (iii) the mean extent of reaction along the chord CDE can be obtained by integration with respect to x, and would be the measured value if light were confined to this path.

(i) *Time distribution over a cross-section.* The velocity distribution for turbulent flow is given by $v = 1{\cdot}225\,\bar{v}(1-r/a)^{\frac{1}{7}}$ (Dodge & Thompson, 1937), where $\bar{v}$ is the average velocity calculated from the volume flow rate. Hence the time of arrival of the fluid at P is

$$t = \frac{\bar{t}}{1{\cdot}225(1-r/a)^{\frac{1}{7}}}. \qquad (1)$$

Similarly, for streamline flow Poiseuille's equation gives

$$t = \frac{\bar{t}}{2(1-r^2/a^2)}. \qquad (1a)$$

(ii) *Distribution of extent of reaction.* The fraction of reaction, f, completed in time t is, for a first-order reaction, $(1-e^{-k_1 t})$ and for a second-order reaction, $k_2 bt/(1+k_2 bt)$; k_1, k_2 are velocity constants, and in the second case b is the initial concentration of the reactants. For ease of manipulation, choose arbitrary time units such that $t_{0{\cdot}5} = 1$ in each case, whence $k_1 = \ln 2$ and $k_2 b = 1$, and the above expressions may be replaced by

$$\text{first order} \quad \log(1-f) = -0{\cdot}301t, \qquad (2)$$

$$\text{second order} \quad f = t/1+t. \qquad (2a)$$

Substitution for t from (1) and (1a) will give f as a function of $\bar{t}$ and r for turbulent and streamline flow, respectively, in the observation tube.

(iii) *Mean extent of reaction along any chord.* For a second-order reaction with streamline flow, the fraction of reaction completed in an element of liquid at P is, from (1a) and (2a),

$$f = \frac{\bar{t}}{2\{1-(x^2+y^2)/a^2\}+\bar{t}} = \phi(x).$$

The mean value of this function in the interval $x=0$ to $x=i$ is $i^{-1}\int_0^i \phi(x)\,dx$, and when $i=(a^2-y^2)^{\frac{1}{2}}$, this will give the average extent of reaction along CD (Fig. 3), and therefore, by symmetry, along the whole chord CDE. The solution is

$$\bar{f} = \frac{2{\cdot}3\bar{t}}{4(1-y^2/a^2)^{\frac{1}{2}}(1-y^2/a^2+\bar{t}/2)^{\frac{1}{2}}} \log\frac{(1-y^2/a^2+\bar{t}/2)^{\frac{1}{2}}+(1-y^2/a^2)^{\frac{1}{2}}}{(1-y^2/a^2+\bar{t}/2)^{\frac{1}{2}}-(1-y^2/a^2)^{\frac{1}{2}}}. \qquad (3)$$

This equation gives the extent of reaction indicated by the light absorption along any chord distance y from the axis, and can be evaluated for any values of $\bar{t}$ and y/a. The corresponding true values of f are obtained by substituting $\bar{t}$ for t in equation (2a).

For turbulent flow or a first-order reaction, the above integration is more difficult, and arithmetic integration has been used (cf. Roughton & Millikan, 1936). Suppose that fluid passing down the observation tube be divided into ten concentric cylindrical elements, and imagine that each element moves as a solid block. Let the elements be of equal thickness, so that the cross-section of the observation tube be divided into ten concentric areas having mean radii $a/20$, $3a/20$, $5a/20$, ..., $19a/20$. The time of arrival of each element at the cross-section of observation may be calculated from these mean radii in terms of $\bar{t}$, by equation (1) or (1a); and then from equation (2) or (2a) the mean extent of reaction in each element may be obtained in terms of $\bar{t}$. If now arbitrary values be given to $\bar{t}$, the corresponding actual extents of reaction in each element may be calculated. Finally, a given light path through the observation tube, along a chord of the cross-section, will pass through some or all of the ten elements, and the length of the path in each element may be calculated geometrically. If Beer's law be valid, the total extinction will be the sum of the extinctions for each element, and the measured percentage reaction along the chord will be the sum of (% reaction × path length) for all the elements, divided by the total path length. For streamline flow and a second-order reaction, this method gives results in close agreement with those obtained from equation (3).

The results of such calculations for the two extreme light paths in the present apparatus, a diameter and a chord bisecting a radius, are given in Table 2. For a first-order reaction and with turbulent flow, these extreme values for the extent of reaction do not differ by more than 2·2%. It may be assumed for the purposes of approximate calcu-

Table 2. *Calculated values for the observed extent of* (1) *a first-order reaction and* (2) *a second-order reaction with streamline and turbulent flow in the observation tube*

(The limiting values are for imaginary light paths confined to a diameter and a chord bisecting a radius: see text.)

True % reaction	Observed % reaction: Streamline flow: Limiting values: Diameter	Streamline flow: Limiting values: Chord	Streamline flow: Mean	Streamline flow: Error	Turbulent flow: Limiting values: Diameter	Turbulent flow: Limiting values: Chord	Turbulent flow: Mean	Turbulent flow: Error
			(1) First-order reaction					
12·9	14·5	18·1	16·3	+3·4	12·2	12·8	12·5	−0·4
29·3	29·7	36·2	33·0	+3·7	27·8	29·1	28·5	−0·8
50·0	46·6	55·2	50·9	+0·9	47·6	49·6	48·6	−1·4
75·0	67·0	76·0	71·5	−3·5	72·3	74·5	73·4	−1·6
87·5	78·8	86·6	82·7	−4·8	85·3	87·0	86·2	−1·3
			(2) Second-order reaction					
9·1	10·0	12·4	11·2	+2·1	8·6	9·0	8·8	−0·2
33·3	32·0	37·9	35·0	+1·7	31·8	33·1	32·5	−0·8
50·0	46·6	53·6	50·1	+0·1	48·2	49·8	49·0	−1·0
66·6	62·3	68·6	65·5	−1·1	65·0	66·4	65·7	−0·9
80·0	76·0	80·9	78·5	−1·5	78·7	80·7	79·7	−0·3

lation that the reaction mixture is illuminated by a parallel beam of light of uniform intensity, in which case the observed percentage reaction should be close to the mean of these extreme values. The data of Table 2 show that for turbulent flow the maximum error calculated in this way is −1·6%. For streamline flow, the discrepancies are greater, and are in opposite senses in the early and late stages of the reaction. For a second-order reaction, the calculated errors are somewhat smaller, the maximum values being +2% and −1% for streamline and turbulent flow, respectively. It should be emphasized that these calculated errors are probably overestimates, since they are derived on the assumption that the characteristic velocity distribution is set up at the entrance to the observation tube, whereas in practice mass flow prevails near the inlet and only gradually gives way to the typical distribution.

It is concluded that with turbulent flow in the observation tube, the method will give valid results, but with streamline flow significant discrepancies might occur, especially with a first-order reaction. These conclusions have been tested to some extent experimentally by comparing kinetic measurements at flow rates above and below the critical velocity (cf. Hartridge & Roughton, 1923), as described later.

Rate and stability of flow

The constancy of the flow rate, which determines the precision of the time measurements, was tested in 'dummy' kinetic experiments with a rotameter and by repeated measurements of the volume of fluid delivered in a measured time. With 0·5 mm. jets, flow rates of from 3 to 7 ml./sec. were obtained in the pressure range 10–40 lb./sq.in. During flow from a single jet, the rotameter readings were quite steady, but with both jets delivering fluid together the readings fluctuated quite rapidly over a range of ±1%. The flow rates calculated from the volume of fluid delivered in periods of 10 sec. or more were constant during an experiment to within ±1%. The upper limit to the flow rate with this jet unit, however, was set at about 5 ml./sec. by the onset of cavitation of flow in the jets, accompanied by noise, slight turbidity and often by a sudden change of the relative rates of delivery through the jets. With 1 mm. jets, the flow was apparently more stable. The rotameter readings were constant to within ±0·2%, and the measured volume flow rate to less than ±0·5%. Cavitation in the jets began at about 15 ml./sec. with a driving pressure of 40 lb./sq.in.

Fluctuations of the relative delivery rates, a possible source of error in the concentration measurements, were tested by mixing 2 N-H_2SO_4 and 2 N-NaOH in the apparatus, and titrating the excess acid or alkali in small samples of the runnings. With both 0·5 and 1 mm. jets, the average relative deliveries for periods of 0·5 sec. and longer did not vary by more than ±0·3% during an experiment.

The slight pulsation of flow indicated by the rotameter was confirmed by vibration of the Beckman galvanometer needle during extinction measurements on a flowing reaction mixture. Similar but smaller variations of extinction reading in absence of any reaction, that is when inert coloured and colourless fluids were mixed in the apparatus, showed that the relative delivery rates also oscillate rapidly over a small range of values. With 0·5 mm. jets, the galvanometer fluctuations did not seriously affect the precision of the measurements (at minimum Beckman sensitivity), as shown by the agreement between duplicate measurements, and it is estimated that the mean extinction reading is subject to an error corresponding to a variation of ±0·5% in the relative delivery rates. With 1 mm.

jets, the instability was more serious, but it was found possible to reduce it by putting a short length of capillary tube at the entrance to the siphon tube in each reactant vessel. With 2·5 cm. lengths of 1 mm. diameter capillary, the galvanometer fluctuations were no greater than with a 0·5 mm. jet unit, and for driving pressures of 10–40 lb./sq.in. the flow-rate range was 5–12 ml./sec. With 1·27 cm. lengths of 0·5 mm. capillaries, the galvanometer fluctuations were almost negligible, and the flow-rate range within the same pressure limits was 2·5–5 ml./sec. The additional resistance to flow is an advantage for work at low flow rates, since the effect of small pressure changes on the flow rate is least at high pressures.

Measurement of concentration

The applicability of Beer's Law. Extinction measurements in a cylindrical tube are liable to errors of two sorts, manifest as apparent deviations from Beer's Law in the form $\log I_0/I_t = kcd$. First, the light path involves refraction, and the amount of light reaching the photocell may change with the refractive index of the solution, and therefore with solute concentration, independently of any absorption. Secondly, different parts of the light beam will traverse different depths of solution, and since I_t, the intensity of the transmitted light, is an exponential function of the product cd, $\log I_0/I_t$ will not be a linear function of c. To minimize such deviations, the observation tube cover was so designed that light can pass only through the middle part of the cross-section of the tube: an approximately parallel light beam from the monochromator falls normally on the 1 mm. slit in the cover, and the observation tube, external diameter 7 mm. and internal diameter 2 mm., is accurately aligned with its axis along the centre of the slit. With water in the observation tube, the light transmission varied by ± 5% over the length of the tube. Geometrically, the longest and shortest possible light paths through the tube differ by 15%, and refraction should be quite small.

The applicability of Beer's law to measurements in the tube was tested with oxyhaemoglobin solutions, which are known to obey the Beer-Lambert law up to high concentrations (Drabkin & Austin, 1935–6). Within the instrumental error, the relation between extinction and concentration was linear up to $E = 1{\cdot}0$–$1{\cdot}5$ at 415, 430, 465, 540 and 560 mμ., and the effective optical depth was constant to within 1% over the length of the tube. Similar results were obtained in a 10 mm. bore tube with a 5 mm. aperture, which is used for stopped flow measurements. So far as errors due to non-uniform optical depth are concerned, these results should be of general validity for the stated geometrical conditions.

The existence of refraction effects was shown by a greater light transmission with solutions of colourless inorganic salts in the observation tube than with water. The transmission increments for 0·1 M solutions were about 0·5%, equivalent to an extinction of −0·002, and increased with concentration. Kinetic measurements would not be significantly affected except at rather high reactant concentrations, and then chiefly through the difference in total solute concentration between the reactant mixture and the 'unreacted' calibration fluid, which lacks one reactant. This could be compensated by an inert solute.

Slit length error. The measured extent of reaction at a given distance from the mixing chamber is really an average value for a small but finite length of the observation tube (the slit length), equivalent to a finite time interval, and for non-linear kinetic curves differs from the actual value at the measured time, the time corresponding to the centre of the slit. Chance (1940) showed that the error depends upon the ratio of slit length to distance from the mixing chamber, and on the stage of the reaction. It is not a limiting factor in the present apparatus: a slit length of only 0·5 mm. gives enough light for measurement, and both calculation and experiment (see later) show that the slit length can be increased to 5 mm. without affecting the results. With a slit length of 2 mm., a spectral band width of 5 mμ. is required at a wavelength of 430 mμ.

Optimum wavelength. The factors which determine the accuracy of the concentration measurements are discussed in the Appendix. The relative magnitudes of the molar extinction coefficients of reactant and product, ϵ_R and ϵ_P respectively, at the wavelength chosen for measurements are shown to affect the accuracy in three ways: (1) the precision of the measurements depends upon the magnitude of $\Delta E_{100\,\%} = C_o d\,(\epsilon_R \sim \epsilon_P)$, the extinction change for complete reaction, and the wavelength should be chosen with regard to the initial reactant concentration, C_o, and the optical depth, d, to give the optimum value of $\Delta E_{100\,\%} = 0{\cdot}8$; (2) the variation of the proportional error with the reactant concentration, C_t, depends upon the sign of $(\epsilon_R \sim \epsilon_P)$, and is least when $\epsilon_R > \epsilon_P$, although smaller errors are obtained over the first half of the reaction when $\epsilon_P > \epsilon_R$; (3) errors due to uncertainties in the relative delivery rates increase with the ratio, r, of the smaller extinction coefficient to the difference between them, and are minimal when the wavelength is such that either ϵ_R or ϵ_P is zero.

Accuracy. With a Beckman spectrophotometer at minimum sensitivity, and the optimum value of $\Delta E_{100\,\%} = 0{\cdot}8$, the fractional composition of the mixture can be determined to within ± 1% C_o or less at all stages of the reaction, so far as the precision of the spectrophotometer is concerned. Over the range $\Delta E_{100\,\%} = 0{\cdot}2$–$1{\cdot}2$, the maximum error is 1·6% C_o, so that at a given wavelength a sixfold variation of the initial reactant concentration is possible without significant loss of precision. These

instrumental errors could be halved by working at maximum spectrophotometer sensitivity, but galvanometer fluctuations due to rapid oscillation of the relative delivery rates nullify the increased sensitivity except at low flow rates. The analytical error resulting from this flow instability depends upon the extinction coefficients of reactant and product (Appendix).

In studying a first-order reaction, it is desirable that the absolute error in log y (where $y = C_t/C_o$), and therefore the proportional error in y, be independent of y. This is most closely approached when $\epsilon_R > \epsilon_P$; if ϵ_P is small, and $\Delta E_{100\,\%} = 0{\cdot}8$, it is estimated that the accuracy of the analysis will be $\pm 1{\cdot}5\,\%$ y over the first 70 % of the reaction, and $\pm 2{\cdot}5\,\%$ y when reaction is 95 % complete.

Temperature control

For work near room temperature, the temperature of the reactants remains quite constant in the lagged pressure chamber, and there is little heat exchange during flow through the lagged leads and the observation tube: if the air temperature is within about 1° of the intial reactant temperature, the temperature of the fluids does not change by more than 0·1°. For stopped-flow measurements, which might incur more serious temperature drifts, a water-jacketed extension to the observation tube is used. Thermostatic control has not yet been attempted, but for work above room temperature heating elements have been inserted in the observation tube cover.

2. THE KINETICS OF CARBONIC ACID DEHYDRATION

The dehydration of carbonic acid has been investigated by several workers with various rapid reaction techniques, and serves as a convenient overall test of the present apparatus. Faurholt (1924) found that the kinetics of $H_2CO_3 \rightarrow CO_2 + H_2O$ at pH < 8 could be described by

$$d\,[CO_2]/dt = k_1\,[H_2CO_3] - k_2\,[CO_2].$$

Brinkman, Margaria & Roughton (1933) showed that if the reaction is brought about in acid solution by mixing sodium bicarbonate and hydrochloric acid, this mechanism should give an approximately linear plot of pH against time, with a slope of $k_1/2{\cdot}3$, and by colorimetric pH measurements in a rapid reaction apparatus, they confirmed Faurholt's conclusions and obtained a mean value of $k_1 = 12{\cdot}9$ sec.$^{-1}$ at 18°, in satisfactory agreement with the results obtained by other methods (Roughton, 1936). The reaction has been studied under the same conditions with the present apparatus.

Experimental

Spectrophotometric measurements of pH. Brode (1924) showed that the effect of pH upon the spectral absorption of several indicators is consistent with the existence of an acid-base equilibrium between two species possessing characteristic absorption spectra, and that pH can be determined accurately by spectrophotometric analysis.

If, for a given wavelength, optical depth and indicator concentration, E_A and E_B are respectively the extinctions of the pure acid and alkaline forms of the indicator (i.e. of calibration fluids of extreme pH), and E is the extinction of the test solution, then assuming that $E_A > E_B$, the ratio of the concentrations of the acid and alkaline forms of the indicator in the test solution is

$$(E_A - E)/(E - E_B) = \{(E_A - E_B) - (E - E_B)\}/(E - E_B).$$

This ratio can be determined most precisely by direct measurement of the extinction differences

$$(E_A - E_B) = \Delta E_{100\,\%} \quad \text{and} \quad (E - E_B) = \Delta E \text{ (Appendix).}$$

Hence,
$$\mathrm{pH} - \mathrm{p}K_{In} = \log \frac{\Delta E_{100\,\%} - \Delta E}{\Delta E},$$

where K_{In} = indicator constant.

The validity of this equation for bromophenol blue was confirmed with Clarke & Lubs phthalate-NaOH and phthalate-HCl buffers of known pH (Vogel, 1939). Potassium hydrogen phthalate, A.R., was dried for 2 hr. at 120°, and conc. NaOH solution made from A.R. sticks was centrifuged to remove carbonate and diluted with CO_2-free distilled water. The extinction measurements were made in the 2 mm. observation tube of the kinetic apparatus, with light of wavelength 435 mμ., the absorption maximum of acid bromophenol blue. The calibration fluids were 0·1 N-HCl and Clarke and Lubs KH_2PO_4-NaOH buffer of pH 7·0. With an indicator concentration of 0·005 % (w/v) the extinction change from acid to alkaline indicator was 0·35, and the precision of the measurements with the spectrophotometer at minimum sensitivity was better than ±0·01 pH unit near $\mathrm{p}K_{In}$.

The plot of log $(\Delta E_{100\,\%} - \Delta E)/\Delta E$ against pH is shown in Fig. 4. Within the experimental error, the points fall on a straight line of slope 0·98, giving $\mathrm{p}K_{In} = 3{\cdot}97$ at an ionic strength of 0·05; Kolthoff & Rosenblum (1937) considered 4·00 to be the best value at this ionic strength.

Kinetic measurements. The reactants were 0·02 M-$NaHCO_3$ and 0·01 N-HCl solutions, each of which contained 0·005 % bromphenol blue, and also served as calibration fluids. At each point of observation, the spectrophotometer was set to zero extinction with the $NaHCO_3$ reagent in the observation tube, and measurements of $\Delta E_{100\,\%}$ and ΔE_t were made on the HCl reactant and the reaction mixture respectively. (Since only 99·2 % of the indicator is in the acid form in the HCl reactant, a correction of 0·8 % was added to the $\Delta E_{100\,\%}$ readings.) In most experiments, kinetic measurements were made at 8 or 10 points along the observation tube, 4 or 5 at increasing distances from the mixing chamber, and the remainder at intermediate points at decreasing distances from the mixing chamber, in order to compensate for small drifts of reactant temperature.

In the pH range of these experiments, the hydration reaction may be disregarded, and the mechanism

$$d\,[CO_2]/dt = k_1\,[H_2CO_3]$$

leads to the relation (Brinkman *et al.* 1933):

$$k_1(t_2-t_1)=\left\{1+\frac{K_{H_2CO_3}}{b-a}\right\}\ln\frac{[H^+]_1}{[H^+]_2}+\left\{1-\frac{K_{H_2CO_3}}{b-a}\right\}\ln\frac{[H^+]_1+b-a}{[H^+]_2+b-a},$$

where a = initial HCl concentration, b = initial $NaHCO_3$ concentration, $K_{H_2CO_3}=[H^+][HCO_3']/[H_2CO_3]$. Since the jets delivered at about equal rates, $a=0{\cdot}005$ and $b=0{\cdot}01$.

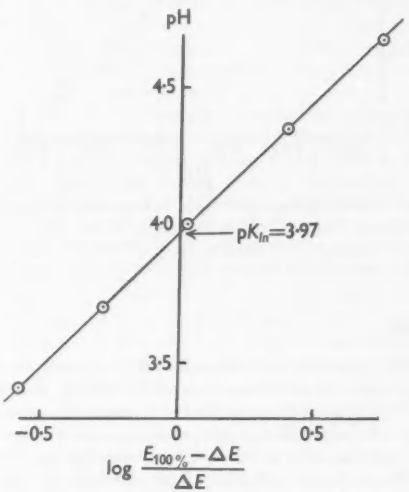


Fig. 4. Calibration curve for spectrophotometric determination of pH with bromophenol blue: plot of pH against $\log\frac{[\text{alkaline indicator}]}{[\text{acid indicator}]}$ for standard buffer solutions.

Taking $K_{H_2CO_3}=3\times10^{-4}$ at 18° (Roughton, 1941) gives $K_{H_2CO_3}/b-a=0{\cdot}06$, and since the ionic strength changes little during the reaction, the above equation approximates very closely to

$$k_1=2{\cdot}30\times1{\cdot}09\,\frac{pH_2-pH_1}{t_2-t_1}. \qquad (4)$$

Results

In Fig. 5, the time course of the pH is shown, as a plot of (pH–pK_{In}) against time, for four successive experiments with a 0·5 mm. jet unit at a flow rate of 4·8 ml./sec. The points fall very closely on straight lines from which only the earliest measurements at a distance of 1 cm. from the mixing chamber deviate by more than the calculated precision of the measurements. The close agreement between pairs of experiments at the same temperature shows that the results are independent of the slit length from 0·5 to 5 mm.

The effect of flow rate is shown in Fig. 6, which gives the results of four experiments with a 1 mm. jet unit at the same temperature of 18·8 ± 0·1°. The

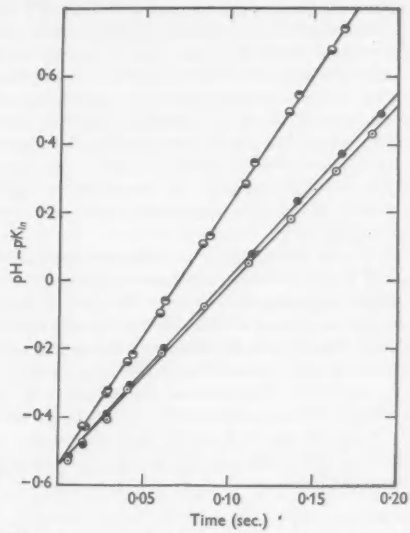


Fig. 5. Time course of pH after mixing $NaHCO_3$ and HCl: ⊙, Exp. 1, 18·9°, slit length 0·5 mm.; ●, Expt. 2, 19·1°, slit length 2 mm.; ◒, Expt. 3, 22·9°, slit length 5 mm.; ◓, Expt. 4, 22·8°, slit length 0·5 mm.

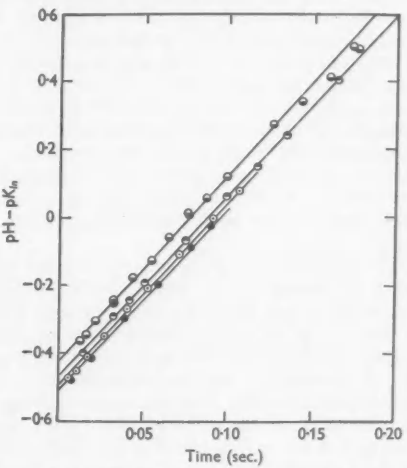


Fig. 6. Effect of flow rate on kinetic measurements: ◒, 2·2 ml./sec.; ◓, 5·3 ml./sec.; ⊙, 8·1 ml./sec.; ●, 10·1 ml./sec.

flow rates were 2·2, 5·3, 8·1 and 10·1 ml./sec., and the critical flow rate for turbulence in the 2 mm. bore observation tube was 3·5 ml./sec. The differences between the intercepts on the ordinate are due to small differences between the relative rates of delivery. The slopes of the best straight lines agree

to within ± 2%, but in the experiment at 2·2 ml./sec. there are significant deviations from linearity in the late stages of the reaction. The deviations are in the sense anticipated theoretically for streamline flow, but are of very much smaller magnitude than the calculated errors. Thus, over the first half of the reaction, the calculated errors (Table 2) are equivalent to no more than 0·02 pH units, but when reaction reaches 87·5% of completion (pH–$pK_{In} = 0·5$) the calculated error of −4·8% amounts to −0·15 pH units.

For fifteen experiments at temperatures in the range 18·8–22·9°, values of k_1 were calculated from the slopes by equation (4), and the plot of log k_1 versus the reciprocal of the absolute temperature is shown in Fig. 7, which illustrates the precision of the method. The radius of the circles is equivalent to 1% k_1 and 0·1°. The greatest deviation from the best straight line amounts to 3% k_1. Extrapolation gives k_1 at 18° as 12·3 sec.$^{-1}$, and the energy of activation for the reaction calculated from the slope is 16 900 cal./mole.

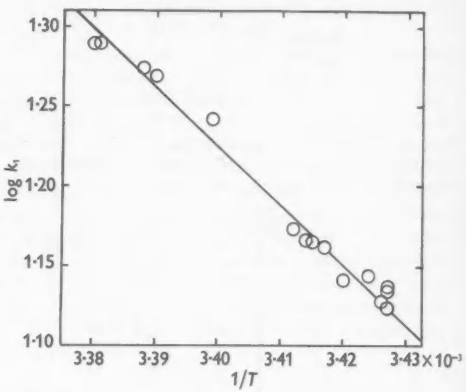


Fig. 7. Effect of temperature on the rate of dehydration of carbonic acid: plot of the logarithm of the velocity constant against the reciprocal of the absolute temperature. The radius of the circles is equivalent to 1% k_1 and 0·1°.

DISCUSSION

For the study of rapid reactions in general, the constant-flow method seems to be potentially more accurate than the accelerated-flow method. Chance's powerful technique was developed especially for the study of enzyme reactions, in which the technical difficulties are greatly augmented by the limited amounts of material available, and often by the smallness of the extinction coefficients of the reactants. For reactions free from these restrictions, the constant-flow method has the advantage of simplicity and of more direct and independent measurements of time and concentration, valuable assets when known and controlled precision is sought.

Of the two types of fluid drive which have been used in the constant-flow method, the original gas-pressure drive and Millikan's motor-syringe drive, the former was chosen as likely to give the more constant flow rate, and also to preclude the possibility of electrical and mechanical interference with the spectrophotometer. The flow rate is constant in the present apparatus to better than ± 1% compared with ± 3% reported by Millikan (1932, 1936). The fluid requirements are of course considerably greater than in Millikan's method; 1 l. of each reactant and 500 ml. of each calibration fluid will give a kinetic curve of 16 points for a reaction of half-period 0·03 sec. These requirements can be halved, with some sacrifice of precision, by modifications of procedure, such as calculation of the flow rate from the pressure and calibration of the observation tube so that only one measurement need be made on the fully reacted calibration fluid.

The principal new feature of the work is the application of accurate spectrophotometric analysis to the constant-flow method by means of a common type of spectrophotometer, with considerable gains in precision and in the range of application of the method. Since square tubes of uniform cross-section are difficult to obtain, and in any case would raise new problems of flow, it was necessary to establish the conditions under which valid measurements of extinction could be made in a cylindrical vessel, in order that Beer's Law could be applied and calibration curves avoided. It is perhaps worth mentioning that the conclusions reached have application to the common test-tube colorimeter. With a Beckman DU spectrophotometer, changes of reactant concentration of less than 1% can be recorded over the first 70% of a reaction, under suitable conditions. With a glass observation tube, any wavelength in the region 350–1000 mμ. is available, and the initial reactant concentrations can therefore be varied considerably without loss of precision; kinetic studies of a reaction at several wavelengths may reveal complexities, and the absorption spectra of transient intermediate compounds can be measured quantitatively (Dalziel & O'Brien, 1952*a*).

The limiting error of the method at present is caused by rapid oscillation of the relative rates of delivery through the jets, manifest as an unsteadiness of the galvanometer needle which limits the precision of the concentration measurements, and prevents full advantage being taken of the sensitivity of the spectrophotometer. Such flow in-

stability is probably inherent in a system of two directly impinging jets with a non-rigid fluid drive. The magnitude of the fluctuations does not change with the flow rate, but increases with the diameter of the jets. The fluctuations with 1 mm. jets were reduced considerably by putting a short length of capillary at the entrance to the leads to the jets. The improvement is due, in part at least, to the fact that higher pressures are then required for a given rate of flow. The available range of flow rates, which is determined by the onset of cavitation in the jets, is greater with this arrangement than with 0·5 mm. jets. It is possible that tangentially opposed jets would give a more stable flow.

The lower limit of the time range of the apparatus in the form described is at most 0·005 sec., the time required for 99% mixing with 1 mm. jets at the highest rate of flow tested, namely 280 cm./sec. in a 2 mm. bore observation tube with a driving pressure of 20 lb./sq.in. With 40 lb./sq.in., a flow velocity of 400 cm./sec. is obtained, and mixing might be still more rapid. More efficient multijet mixers might be used, as in earlier types (Roughton & Millikan, 1936). The interaction of the various factors which determine the efficiency and range of the method have been discussed by Millikan (1936) and by Chance (1940).

The conclusions drawn from control experiments and calculations regarding the precision and validity of the method are supported by experimental work on the dehydration of carbonic acid. Measurements at several rates of flow conformed closely to the requirements of the accepted mechanism, and estimates of the velocity constant were reproducible to $\pm 3\%$. The mean value of $k_1 = 12{\cdot}3$ sec.$^{-1}$ at 18° is in satisfactory agreement with those of most previous workers, listed by Roughton (1936, 1941), and the figure of 16900 cal./mole for the activation energy agrees well with the value of 16500 cal./mole obtained by Roughton (1941) with a thermal method. With the indicator method used in the present work, Brinkman *et al.* (1933) found $k_1 = 12{\cdot}8$ sec.$^{-1}$ at 18° as the mean of two results differing by 10%; using the data of a third, unpublished, experiment Roughton (1941) corrected this figure to 13·6 sec.$^{-1}$. With the thermal method, however, Roughton (1941) obtained $k_1 = 14{\cdot}6 \pm 0{\cdot}7$, which he considers to be the most reliable value. There is a possibility that the indicator method may be unsuitable for rapid reactions involving carbon dioxide (Roughton, 1952).

Further evidence for the precision of the technique was obtained in studies of the deoxygenation of human haemoglobin (Dalziel & O'Brien, 1950) with an earlier version of the apparatus. The difference between duplicate measurements at the same distance along the tube in a large number of experiments very rarely exceeded the estimated error of time and concentration measurements, and in a set of four experiments with the same sample of blood under identical conditions the variation in the velocity constant for the last 70% of the reaction was $\pm 2\%$. An upward trend of the velocity constant during the first 30% of the deoxygenation, predicted by the intermediate compound theory (Roughton, 1949) and recently detected by Legge & Roughton (1950), was confirmed in these experiments at wavelengths of 415, 430 465 and 560 mμ., and lies well outside the error of analysis. It is hoped that the technique will yield kinetic data accurate enough to contribute to the experimental evaluation of the intermediate compound theory outlined by Roughton (1949) and developed in recent accurate equilibrium studies (Paul & Roughton, 1951; Lyster, Otis & Roughton, 1951).

SUMMARY

1. A form of Hartridge-Roughton rapid reaction apparatus has been developed with precision as the major objective of the design. The apparatus consists of a constant-flow apparatus with gas-pressure drive and a photoelectric spectrophotometer with which the extent of a reaction or the spectral absorption of transient compounds may be measured.

2. The conditions under which Beer's law may be assumed valid for extinction measurements in a cylindrical tube have been determined, and the factors governing the choice of wavelength for accurate kinetic measurements are stated. The extent of a suitable reaction can be estimated to within $\pm 1{\cdot}5\%$ or less over the first three-quarters of its course, the limiting error arising from the instability of the relative rates of delivery of the reactants through the jets.

3. It is shown theoretically that with turbulent flow in the observation tube the validity and precision of the method should not be affected significantly by the deviations from ideal flow. The precision of the time measurements, determined by the constancy of the total flow rate, is $\pm 1\%$.

4. At the highest flow rate tested, 280 cm./sec. in the 2 mm. bore observation tube with a driving pressure of 20 lb./sq.in., mixing occurs to the extent of 99% in 0·005 sec.

5. A value of $12{\cdot}3 \pm 0{\cdot}4$ sec.$^{-1}$ at 18° was obtained for the specific reaction rate of

$$H_2CO_3 \rightarrow CO_2 + H_2O$$

by spectrophotometric measurements of the rate of change of pH in acid-bicarbonate mixtures. The effect of temperature on the velocity constant in the range 19–23° gave the energy of activation as 16900 cal./mole. The pK_{In} value for bromophenol blue was found to be 3·97 at an ionic strength of 0·05.

I wish to thank Mr J. R. P. O'Brien, at whose suggestion this work was undertaken, for advice and criticism. I am very greatly indebted to Mr J. T. Cox, who constructed the apparatus and contributed much to the design, and to Mr H. Vincent, who rendered similar services in connexion with the glass components. I am grateful also to Mr B. A. Collett for technical assistance during part of the work, to Mr R. S. Salt for help with the gas-pressure system and to Dr G. H. Epstein for helpful discussions of flow problems.

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APPENDIX

The Precision of Analysis by Photoelectric Spectrophotometry

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Twyman & Lothian (1933) showed that the percentage error of photoelectric measurements of extinction, E, is at a minimum for $E = 0{\cdot}43$. If $\delta\theta$ = smallest detectable galvanometer deflexion, T = transmission = I_t/I_o and δT = smallest measurable transmission change, then $\delta T = k\delta\theta$, where k is constant for a given voltage drop across the potentiometer. Since

$$E = \log 1/T, \quad dE = -dT/2{\cdot}3T = -dT\ 10^E/2{\cdot}3,$$

and hence, if δT is small, the smallest measurable extinction change is

$$\delta E = -\frac{10^E}{2{\cdot}3}\,\delta T. \qquad (1)$$

The precision of extinction measurements therefore decreases with increase of extinction. The proportional error of extinction measurements is given by

$$p = \frac{\delta E}{E} = -\frac{10^E}{2{\cdot}3E}\,\delta T, \qquad (2)$$

and is a minimum when $E = 0{\cdot}43$.

With the Beckman spectrophotometer at minimum potentiometer sensitivity, we find

$$\delta T = 2 \times 10^{-3}.$$

The corresponding absolute and proportional errors of extinction measurements, calculated from equations (1) and (2), respectively, are plotted against extinction in Fig. 1: if Beer's law is obeyed, these curves show the errors in concentration measurements. At maximum sensitivity, $\delta T = 10^{-3}$, and the errors are half as great.

If a spectrophotometer be set to read $E = 0$ with an absorbing solution A, the reading with a second solution B is the extinction difference, $\Delta E = E_B - E_A$. Equations (1) and (2) will apply if ΔE is substituted for E, and it follows that, for a given value of δT, a more precise estimation of ΔE will be obtained in this way than by measuring E_A and E_B separately. This principle can be used, for example, to determine accurately the ratio of the optical depths of two cells.

Analysis of reaction and equilibrium mixtures

For an isolated or reversible reaction in which reactant R is transformed directly into a single stable product P, the extinction of the reacting mixture at any wavelength for which R and P are the only absorbing species is given by

$$E_t = \epsilon_R C_t d + \epsilon_P (C_o - C_t) d,$$

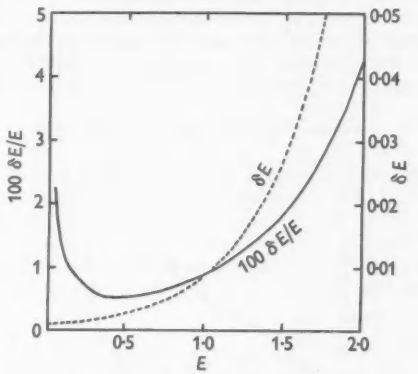

Fig. 1. Calculated precision of extinction measurements with a Beckman spectrophotometer at minimum sensitivity; δE = smallest detectable extinction change.

where C_o = initial molar concentration of R, C_t = concentration of R at time t, d = optical depth, and ϵ_P, ϵ_R = molar extinction coefficients of R and P. The fraction y of reactant remaining at time t is

$$y = \frac{C_t}{C_o} = \frac{E_t \sim \epsilon_P C_o d}{(\epsilon_R \sim \epsilon_P)\, C_o d} = \frac{E_t \sim E_P}{E_R \sim E_P}, \qquad (3)$$

where E_R and E_P are the extinctions of solutions of R and P of concentration C_o, measured at the same optical depth and wavelength as E_t. If such solutions are used as calibration fluids, the calculation of y will be independent of the optical depth and the wavelength, and the accuracy of the analysis will depend only upon (i) the precision of the measurements, and (ii) the accuracy with which the reaction mixture and the calibration fluids are made up. For a reversible reaction, equation (3) with $E_t = E_\infty$ will give the composition of the mixture at equilibrium, and is applicable, for example, to the spectrophotometric determination of pH with indicators and with buffer solutions of extreme pH ($pK_{In} \pm 3$) as calibration fluids.

(i) *Precision of the measurements.* Since extinction differences are obtained most accurately by direct measurement, the spectrophotometer should be set to $E = 0$ with the calibration fluid having the smaller extinction coefficient. The reading with the other calibration fluid will be the extinction change for complete reaction, $\Delta E_{100\,\%}$. If ΔE_t is the reading with the reaction mixture, equation (3) becomes:

$$\text{when} \quad \epsilon_R > \epsilon_P \quad y = \frac{\Delta E_t}{\Delta E_{100\,\%}}, \qquad (4a)$$

$$\text{and when} \quad \epsilon_P > \epsilon_R \quad (1-y) = \frac{\Delta E_t}{\Delta E_{100\,\%}}, \qquad (4b)$$

where $(1-y)$ = fractional concentration of product P.

The absolute error in measuring ΔE_t increases with ΔE_t, according to equation (1). For a given value of $\Delta E_{100\,\%}$, therefore, equations (4*a*) and (4*b*) show that the absolute error in y will increase with y if $\epsilon_R > \epsilon_P$, and decrease with increase of y if $\epsilon_P > \epsilon_R$. The first condition will give the smaller variation of the proportional error with y, and is therefore to be preferred for kinetic measurements over the whole course of a first-order reaction, for which $\log y$ is the significant quantity. This is one factor governing the choice of the most suitable wavelength for the analysis.

For given substances R and P, $\Delta E_{100\,\%}$ is determined by the wavelength, the initial reactant concentration and the optical depth. For the case $\epsilon_R > \epsilon_P$, the absolute error in y, δy, is related to the proportional errors in ΔE_t and $\Delta E_{100\,\%}$, p_t and $p_{100\,\%}$ respectively, by

$$\delta y = y\sqrt{(p_t^2 + p_{100\,\%}^2)}. \qquad (5)$$

Since p is minimum when $\Delta E = 0{\cdot}43$ (Fig. 1), and since $\Delta E_t < \Delta E_{100\,\%}$, equation (5) shows that δy will decrease with increase of $\Delta E_{100\,\%}$ up to

$$\Delta E_{100\,\%} = 0{\cdot}43,$$

for all values of y. Higher values of $\Delta E_{100\,\%}$ will give still smaller errors at low values of y, but greater errors at high values of y. Thus, the optimum value of $\Delta E_{100\,\%}$ varies with y, but is always greater than 0·43.

These considerations are given quantitative expression in Figs. 2 and 3, which show the errors in determining y with a Beckman spectrophotometer at minimum sensitivity, calculated from equation (5) and the data of Fig. 1. The absolute errors in y for three values of $\Delta E_{100\,\%}$, 0·4, 0·8 and 1·2, are shown in Fig. 2 as a plot of $100\delta y$ against $100y$: the curves show the absolute errors in measuring the percentage composition of equilibrium mixtures or the absolute errors in measuring the concentration of reactant ($\epsilon_R > \epsilon_P$) or product ($\epsilon_P > \epsilon_R$) expressed as a percentage of the initial reactant concentration. It can be seen that for the highest precision, the wavelength and the optical depth should be chosen with regard to the initial reactant concentration to give $\Delta E_{100\,\%} = 0{\cdot}4$–$0{\cdot}8$, when the composition of any mixture can be determined with a precision of $\pm 1\,\%$ of the initial reactant concentration.

For kinetic measurements, the proportional errors in y, $\delta y/y$, are of more significance than the absolute errors. The most uniform distribution of proportional errors over the whole course of a reaction is obtained when $\epsilon_R > \epsilon_P$ and $\Delta E_{100\,\%} = 0{\cdot}8$.

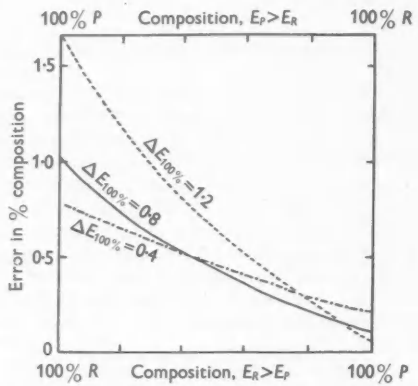

Fig. 2. Precision of measurements of the composition of equilibrium or reaction mixtures of substances R and P, for various values of $\Delta E_{100\,\%} = E_R \sim E_P$, the extinction change for complete conversion of R to P.

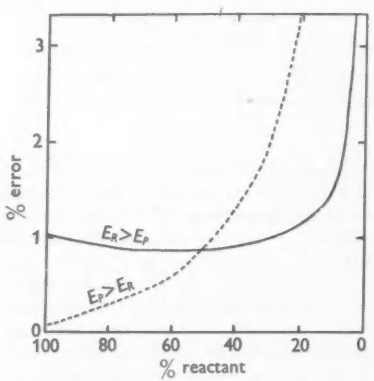

Fig. 3. Percentage errors of measurements of the fractional concentration of reactant R in a reaction or equilibrium mixture, with the optimum value of the extinction change for complete reaction, $E_R \sim E_P = 0{\cdot}8$.

These conditions also give the minimum proportional errors over the second half of the reaction; for the first half, the errors are smaller when $\epsilon_P > \epsilon_R$ and $\Delta E_{100\,\%} = 0{\cdot}8$. The calculated percentage errors in y as a function of y are shown in Fig. 3 for these two cases, again for the Beckman spectrophotometer at minimum sensitivity; at maximum sensitivity the errors are half as great.

(ii) *Effect of errors in the initial reactant concentration.* The validity of equation (4) requires that the concentration of each calibration fluid be equal to the initial concentration of the reaction mixture. This condition has practical significance for the flow method, in which the initial reactant concentration depends upon the relative delivery rates through the jets. It will be shown that, for a first-order reaction, the effect of an uncertainty in this quantity upon the accuracy of the analysis depends upon the choice of wavelength and is determined by the relation between the extinction coefficients of reactant and product.

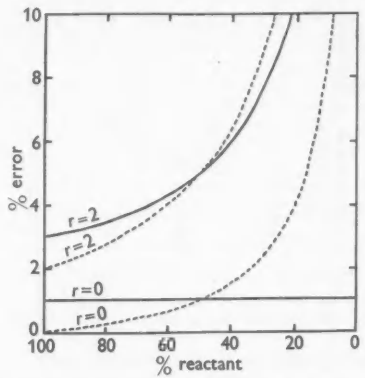

Fig. 4. Percentage errors of measurements of the fractional concentration of reactant R in a reacting mixture resulting from a 1% difference between the concentration of the mixture and that of the calibration fluids. Full lines for $\epsilon_R > \epsilon_P$, broken lines for $\epsilon_P > \epsilon_R$; ϵ_R and ϵ_P = molar extinction coefficients of reactant and product, r = ratio of the smaller extinction coefficient to the difference between them.

Let e = proportional error in the initial reactant concentration, defined by $e = (C_o - C_o')/C_o$, where C_o = concentration of the calibration fluids, and C_o' = initial reactant concentration in the mixture. Since, for a first-order reaction, the fraction of the total reaction completed in a given time is independent of the initial reactant concentration, the extinction of the reaction mixture, E_t, will also suffer a proportional error e at all values of t, i.e. $\delta E_t/E_t = e$.

If the wavelength is such that $\epsilon_R > \epsilon_P$, equation (4*a*) is applicable, viz.

$$y = \frac{\Delta E_t}{\Delta E_{100\,\%}} = \frac{E_t - E_P}{E_R - E_P}. \qquad (6)$$

Differentiating and substituting for y

$$\frac{\mathrm{d}y}{y} = \frac{\mathrm{d}E_t}{E_t - E_P} = \frac{\mathrm{d}E_t}{E_t}\,\frac{E_t}{E_t - E_P}.$$

Hence the proportional error in y resulting from a small proportional error e in E_t is

$$p_y = eEt/(E_t - E_P),$$

and substituting for E_t from (6)

$$p_y = e\left(1 + \frac{E_P}{y(E_R - E_P)}\right) = e\left(1 + \frac{r}{y}\right), \qquad (7)$$

where $r = E_P/(E_R - E_P) = \epsilon_P/(\epsilon_R - \epsilon_P)$, that is, the ratio of the smaller extinction coefficient to the difference between them. Alternatively, if the wavelength chosen is such that $\epsilon_P > \epsilon_R$, it can be shown in a similar way from equation (4*b*) that

$$p_y = e\left(1 - \frac{1+r}{y}\right),$$

where r is again the ratio of the smaller extinction coefficient to the difference between them, in this case $\epsilon_R/(\epsilon_P - \epsilon_R)$.

For given substances R and P, r is determined solely by the wavelength. The error in determining y due to a given error in the initial reactant concentration therefore depends solely on the wavelength at which the measurements are made, and, in contrast to the precision of the measurements, is independent of the initial reactant concentration and the optical depth. The error increases with r, and is minimum when $r = 0$, that is when a wavelength is used which either the reactant or the product does not absorb. In Fig. 4, $p_y e$ is plotted against y for values of r of 0 and 2: the curves show the percentage error in y resulting from a 1% error in the initial reactant concentration, calculated from equations (7) and (8). It is evident that with high values of r errors in the initial reactant concentration are magnified considerably in the result, and in the flow method the constancy of the relative delivery rates will be critical. As in the case of the precision of the measurements, the condition $\epsilon_R > \epsilon_P$ gives the more uniform distribution of errors over the whole course of the reaction, and the smaller errors over the second half of the reaction, whilst the condition $\epsilon_P > \epsilon_R$ is better for the first half of the reaction.

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Mould 'Glucosaccharase': a Fructosidase

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(*Received* 5 *December* 1952)

Bealing & Bacon (1953) have shown that a transfructosidase is probably the only enzyme in extracts of *Penicillium spinulosum* that attacks sucrose. Their experimental data did not exclude the possibility that an additional enzyme attacking sucrose was present in extracts of *Aspergillus oryzae* and *A. niger*.

Further study of the extracts of *P. spinulosum* has shown that the transfructosidase is inhibited by glucose, i.e. it possesses a property previously attributed to a hypothetical α-glucosidase ('glucosaccharase': Kuhn, 1923) and accepted as a criterion to distinguish this enzyme from other invertases. The results presented here suggest that the inhibitory action of sugars and alcohols on mould invertase may be explained by their action as acceptors of fructose residues.

Evidence is also presented that the action of mould extracts on β-fructofuranosides other than sucrose, viz. raffinose, stachyose and melezitose, may be explained without assuming the presence of a glucosaccharase capable of attacking α-glucosidic linkages similar to that found in sucrose.

These observations lead to the conclusion that there is now little reason to presume that mould extracts contain a glucosaccharase: whether or not enzymes of this type do exist remains to be established.

MATERIALS AND METHODS

The organisms, culture media, and method of preparation of extracts have been described previously (Bealing & Bacon, 1953). This procedure was modified in one instance, when a series of Czapek-Dox media was prepared in which the 5% (w/v) sucrose was replaced by raffinose, maltose, glucose, sorbose, fructose or galactose. Each solution was inoculated with spores of *P. spinulosum* (grown on a sucrose medium) and incubated at room temperature (18–24°). Mycelia were harvested at the beginning of active sporulation, after 8 days on the sucrose, raffinose, maltose and glucose media, and after 14 days on the sorbose and fructose media. Material was not obtained from the galactose media because

of the extremely slow rate of mycelial growth. Extracts were prepared in the usual way from the crushed mycelia.

Incubation procedure. Mixtures of buffer, substrate and enzyme were incubated in glass-stoppered tubes at room temperature. The buffers usually employed were sodium acetate-acetic acid (pH 5·0) and KH_2PO_4-Na_2HPO_4 (pH 6·8).

Samples of incubation mixtures were inactivated either by heating for 3 min. in tubes previously placed in a boiling-water bath, or by the addition of $HgCl_2$ solution to give a final concentration of 0·001 to 0·002 M-$HgCl_2$.

Paper-partition chromatography and the estimation of sugars. Chromatograms were prepared by descending development in the butanol:acetic acid:water solvent described by Partridge (1948), using the procedures of Bacon & Edelman (1951), Bacon & Loxley (1952) and Bealing & Bacon (1953).

The methods employed for the estimation of sugars were those of Bealing & Bacon (1953).

Stachyose preparation. A sugar considered to be stachyose, *O*-α-D-galactopyranosyl-(1→6)-*O*-α-D-galactopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→2) β-D-fructofuranoside (Adams, Richtmyer & Hudson, 1943; Hérissey, Wickstrom, Courtois & Dizet, 1952), was isolated from rhizomes of *Stachys sylvatica* L. (hedge woundwort). The rhizomes, dug up in September, were washed, cut into sections 4–5 cm. long, and thrown into water maintained at boiling point for 10 min. An extract was obtained by treatment of this material in a Waring Blendor with the minimum amount of water: after squeezing through madapollam (a cotton cloth supplied by C. H. Baddeley and Co., 7 Buck Street, Leeds Road, Bradford; cf. Bawden & Pirie, 1942) the solution was deproteinized by the addition of 30% (w/v) lead acetate solution until no further precipitate appeared. Excess lead was removed by precipitation with 3% (w/v) sodium oxalate. The extract was concentrated to a tenth of its original volume, and the carbohydrates precipitated by the addition of 4 vol. of ethanol. Partition chromatography on cellulose (Whatman Cellulose Powder 'Standard Grade': *n*-butanol saturated with water as solvent; cf. Hough, Jones & Wadman, 1949) was used to separate the major constituent of this precipitate from other sugars.

The product obtained was an almost colourless glass melting at 165–175° and having $[\alpha]_D^{20}+131°$ (water, $c=0{\cdot}8$). Stachyose has m.p. 167° and $[\alpha]_D+150°$ (anhydrous) (Tanret, 1902).

Diazouracil (2 mg.) was mixed with 33 mg. of this material and 2 ml. 0·05 N-NaOH were added to give an opalescent solution which developed a green coloration after standing for 4–5 min. at 0°. Control experiments gave similar positive results with sucrose and raffinose but not with melezitose, nor in the absence of added carbohydrate. These results are in accordance with the reaction described by Raybin (1933, 1937) for sucrose, raffinose, gentianose and stachyose.

Paper chromatography showed that the glass contained a single oligosaccharide having R_F 0·02 (butanol:acetic acid; compared with sucrose, R_F 0·11). This substance did not reduce alkaline silver nitrate (Trevelyan, Procter & Harrison 1950); it contained fructose.

Partial hydrolysis was achieved by heating with 0·05 N-oxalic acid to 100° for 5 min., followed by rapid cooling, or by the action of yeast invertase. In both cases fructose was liberated. The residual oligosaccharide reduced alkaline silver nitrate and did not contain fructose. More complete hydrolysis was obtained by treatment with 0·5 N-H_2SO_4 at 100°. After 1 hr., fructose had been liberated and the residual sugar partly broken down to a monosaccharide (galactose or glucose) and a second reducing sugar having the R_F value of melibiose. Further heating was accompanied by destruction of fructose so that, when hydrolysis was complete after about 8 hr., the solution contained only galactose and glucose; as judged qualitatively, the mixture contained a higher proportion of the former sugar.

These observations are in accordance with the idea that the carbohydrate isolated was stachyose. The R_F value of manninotriose (0·04 in butanol:acetic acid: Bradfield & Flood, 1950) corresponds with that of the oligosaccharide released by partial hydrolysis. The existence of the 6-α-D-galactosyl-glucose structure in stachyose and manninotriose (Hérissey *et al.* 1952) would indicate that melibiose (6-α-galactosyl-D-glucose) could appear as an intermediate in the hydrolysis of these substances.

Electrophoresis on filter paper. The apparatus used was based on that described by Durrum (1950). It consisted of two glass jars each holding 2 l. of buffer and linked by a strip of Whatman no. 3MM paper 6 in. wide and 24 in. long, the whole being enclosed in a glass tank sealed with a greased glass plate. A graphite electrode was suspended in each jar and 340 v applied between the terminals. Under the conditions used the current passing through the paper was 0·3–1·0 mA.

By the use of this technique a partial separation of the enzymes in mould extracts was accomplished. Volumes of 0·3–0·5 ml. of extract were placed as a band across the strip of paper and allowed to dry at room temperature; the paper was then soaked in the minimum amount of a buffer (10–15 ml.) delivered from a pipette drawn over the surface of the paper.

When 0·01 M-phosphate buffer (pH 6·8) was used, application of 340 v between the ends of the strip resulted in the movement of a sucrose-splitting enzyme at about 2 cm./hr. towards the cathode. Relatively small amounts of enzymic activity remained adherent to the paper at the base-line and behind the main band. Under these conditions maltase activity moved towards the anode, away from the invertase; when electrophoresis was carried out at pH 5·0 (0·01 M-acetate buffer) the invertase and maltase activities migrated together towards the anode.

The position of the invertase could be found by spraying the paper with a sucrose solution, incubating in a damp atmosphere for several hours at room temperature, and determining the position of reducing sugars by treatment with alkaline silver nitrate. More usually, however, the paper was cut into strips about 3 cm. wide; segments of the strips were incubated separately with solutions of sucrose or other sugars and the occurrence of enzymic activity tested by paper chromatography of the solution.

The breakdown of sucrose by the invertase band was qualitatively similar to that resulting from the action of entire mould extracts. Attempts to elute the enzyme were not successful; at pH 6·8 (phosphate) no activity was recovered, and at pH 4·5 (acetate) only 20% of the applied invertase activity was eluted. It appeared improbable that the latter value provided a true estimate of the amount of enzyme present, and it was therefore not possible to determine the percentage of the total sucrase activity which was represented by the separated enzyme; however, the occurrence of a second invertase band was not observed under any conditions.

RESULTS

Inhibitory effects of sugars

Effect of glucose. The effect of glucose on the rate of breakdown of sucrose was investigated by polarimetry.

An extract of crushed *P. spinulosum* mycelium (2·5 ml.) was added to each of two buffered sucrose solutions (12·5 ml.) to give a final concentration of 10·0% (w/v) sucrose in 0·005M-acetate buffer (pH 5·0). One solution contained in addition 5·0% (w/v) mutarotated glucose.

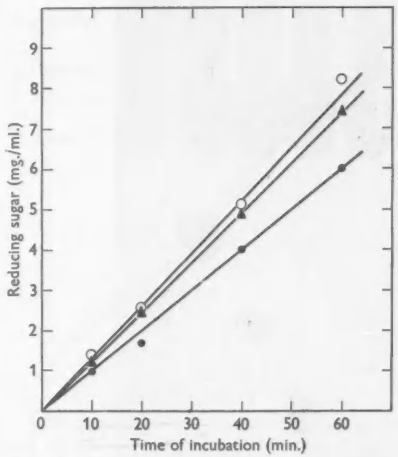

Fig. 1. Inhibitory effect of added glucose and fructose on the rate of inversion of sucrose. Samples of reaction mixtures inactivated by heating and left 12 hr. to permit mutarotation of glucose before measurement of optical rotations. Values for liberation of reducing sugars calculated from polarimetric data, assuming invert sugar to be liberated. ○—○, solution containing 10·0% (w/v) sucrose; ●—●, solution containing 10·0% (w/v) sucrose, 5·0% (w/v) glucose; ▲—▲, solution containing 10·0% (w/v) sucrose, 5·0% (w/v) fructose.

Successive samples of each solution were analysed by polarimetry; the addition of glucose was found to diminish the apparent rate of liberation of reducing sugars (Fig. 1).

The course of sucrose breakdown in solutions containing different amounts of glucose was followed by paper chromatography. Equal volumes (1·0 ml.) of an extract of crushed *P. spinulosum* mycelium were incubated with a series of buffered sucrose solutions (7·0 ml.) containing various amounts of mutarotated glucose (final concentrations, 7·5% sucrose and 0, 3·9, 5·8, or 7·8% glucose in 0·005M-acetate buffer, pH 5·0). Samples taken at intervals were analysed by paper chromatography.

The results of these analyses (Fig. 2) showed that the presence of glucose decreased the rate of sucrose disappearance, there being a corresponding decline in the rate of formation of 'upper spots' (Bealing & Bacon, 1953) but no effect on the liberation of fructose.

Effect of fructose. Fig. 1 shows the inhibitory effect of 5% fructose in the system used to study the inhibition by glucose.

In another experiment, an extract of *P. spinulosum* mycelium (0·4 ml.) was added to each of a series of buffered sucrose solutions (1·6 ml.) which contained different amounts of fructose (final concentrations, 7·5% sucrose and 0, 3·9, 7·8 or 11·7% fructose in 0·006M-acetate buffer, pH 5·0). Samples

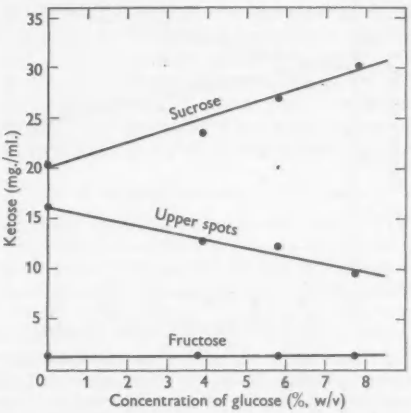

Fig. 2. Effect of added glucose on the breakdown of sucrose by mould extracts. Mixtures incubated at room temperature and sample of each analysed after 1·5 hr. For details see text. Sucrose and upper spots expressed as mg. ketose/ml. On lines at about 4%: (upper line) sucrose, (middle line) upper spots, (bottom line) fructose.

taken after 90 min. were analysed by paper chromatography. The addition of fructose resulted in a decreased rate of disappearance of the ketose of the sucrose spot and a decreased rate of formation of upper spots (Fig. 3). No attempt was made to measure fructose liberation because of the relatively very great fructose 'blank'.

The apparently diminished rate of sucrose breakdown was associated with the appearance of a reducing sugar having an R_F value similar to that of sucrose. This substance was present in incubation mixtures after prolonged incubation of mould extracts with sucrose alone, but its formation was accelerated by the presence of fructose.

The production from raffinose of a reducing substance having approximately the same R_F value as sucrose but containing only fructose is described

below. In this case also, the addition of fructose had an accelerating effect on the rate of formation.

Effect of other sugars. The addition of galactose, mannose, arabinose or xylose to reaction mixtures decreased the rate of liberation of reducing sugars from sucrose by a Taka diastase solution. The solutions were examined by paper chromatography in an attempt to demonstrate the incorporation of the added sugars in oligosaccharides: this was successful only in the case of the mixture containing xylose, in which four new spots were detected. These substances appeared in order of decreasing R_F values.

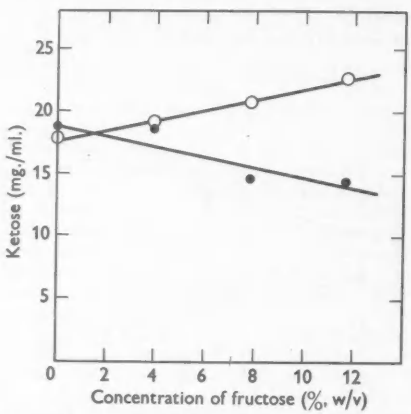

Fig. 3. Effect of added fructose on the breakdown of sucrose by mould extracts. For details, see text. Mixtures incubated at room temperature. Sucrose (○—○) and upper spots (●—●) expressed as mg. ketose/ml.; fructose liberation not measured.

The presence of xylose in each was indicated by a characteristic pink coloration produced by this sugar when sprayed with the benzidine-trichloroacetic acid reagent; confirmation was obtained by hydrolysis (with yeast invertase) of the two faster-running spots separated on a two-dimensional chromatogram.

Inhibitory effects of alcohols

Bacon (1952) has shown that methyl β-fructofuranoside is formed during the action of yeast invertase on sucrose in the presence of methanol. Addition of higher alcohols leads to the appearance of other substances having greater R_F values and considered to be homologous with the methyl fructoside.

Similar, probably identical, compounds were produced by Taka diastase or mould extracts incubated with sucrose (or raffinose) in the presence of methanol, ethanol, *n*-propanol, or *n*-butanol (Fig. 4). Each spot contained fructose (reaction with phloroglucinol), and the R_F values increased with the size of the alkyl group expected to be present; the R_F value of the methanol spot was the same as that of methyl β-fructofuranoside.

The subsequent appearance of a second spot was observed in the solutions containing ethanol, *n*-propanol or *n*-butanol; in each case the R_F value of

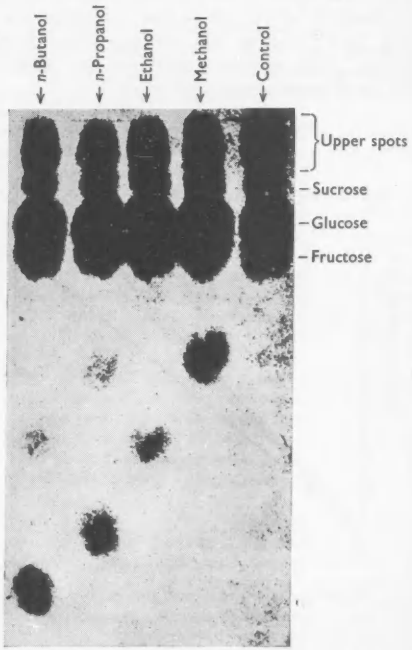

Fig. 4. Formation of fructosides in presence of alcohols. Solutions containing 1·0 ml. 15% (w/v) sucrose, 0·4 ml. water or alcohol, 0·2 ml. 0·05 M-acetate buffer (pH 5·0) and 0·4 ml. extract of *P. spinulosum* mycelium incubated for 8 hr. (Sprayed with benzidine-trichloroacetic acid; negative made by transmitted ultraviolet light.)

these substances was less than that of the first spot, and it is probable that a corresponding substance present in the methanol incubation would be obscured by the sugar spots. Traces of a third substance with a still smaller R_F value could be detected in solutions containing *n*-butanol.

Fructose-containing spots with high R_F values also appeared when ethylene or propylene glycols were added to incubation mixtures, but not when *iso*propanol, *sec.*- or *tert.*-butanol, *n*-amyl, or benzyl alcohols were added.

Hydrolysis of the fructosides occurred after a long incubation period; extracts of *P. spinulosum* were also capable of hydrolysing methyl β-fructo-

furanoside (obtained by the action of yeast invertase on a sucrose-methanol mixture).

Addition of alcohols resulted in a reduced rate of liberation of reducing sugar from sucrose, the effect of methanol in an extract of *A. oryzae* being shown in Fig. 5. Analyses of paper chromatograms showed that the rate of formation of upper spots decreased with increasing concentration of methanol. In the few experiments carried out the amount of methyl fructoside formed did not account for the progressive decrease in the rate of liberation of reducing sugar.

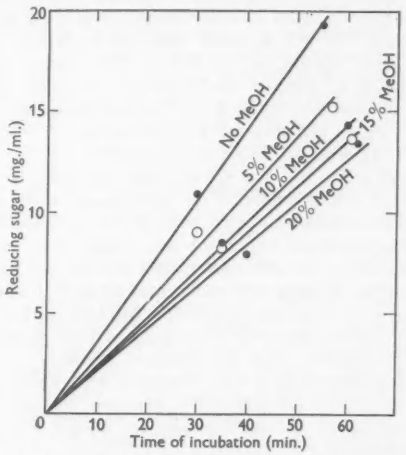

Fig. 5. Liberation of reducing sugar in presence of methanol. Equal volumes (1·2 ml.) of an extract of crushed *A. oryzae* mycelium added to a series of buffered sucrose solutions (2·8 ml.) containing different amounts of methanol to give final concentrations, 7·5% (w/v) sucrose and 0, 5, 10, 15, or 20% (v/v) methanol in 0·05 M-acetate buffer (pH 5·0). Reducing-sugar content of 10 or 20 μl. quantities of each solution measured after approx. 30 and 60 min.

Effect of inorganic phosphate

Samples of a solution containing 1·0 ml. 32% sucrose, 0·5 ml. 5% (w/v) Taka diastase solution and 2·0 ml. 0·2M-phosphate buffer (pH 6·8) were taken after 3 and 6 hr. The mixtures were chromatographed on paper (previously washed with the tetrasodium salt of ethylenediaminetetraacetic acid in water), using ascending development and *tert.*-amyl alcohol: formic acid as solvent (Hanes & Isherwood, 1949). When the chromatograms were sprayed with the perchloric acid-ammonium molybdate reagent of these authors, heated for 5 min. at 85° and treated with H_2S, blue spots indicating the position of inorganic phosphate appeared but the occurrence of sugar phosphates was not detected.

In a second experiment, two incubation mixtures were prepared, one solution containing veronal buffer (pH 7·0) and the other phosphate buffer adjusted to the same pH value. Sodium fluoride was added to the phosphate solution to decrease the hydrolysis by phosphatase of any sugar phosphates which might be formed, but analyses of samples by paper chromatography failed to show that the presence of phosphate influenced the course of the reaction (Table 1). It would thus seem that inorganic phosphate neither functions as an acceptor, nor has any inhibitory effect on sucrose breakdown.

Action of mould extracts on other sugars

Raffinose. All of the preparations examined (from *P. spinulosum*, *A. niger*, *A. oryzae*, *A. flavus*, and Taka diastase) were capable of hydrolysing raffinose. The course of the reaction depended on the presence or absence of melibiase. The adaptive formation of melibiase by *A. niger* has been described by Pottevin (1903); similar observations have been made in the present investigation with *P. spinulosum*. This mould, which had been subcultured on sucrose-containing media for 2 yr., produced insignificant amounts of melibiase when grown on sucrose media, but appreciable amounts of this enzyme when grown on raffinose or glucose media.

When melibiase activity was absent, raffinose was hydrolysed to a mixture of fructose and melibiose. The reaction was accompanied by the appearance of a fructose-containing substance ('first upper spot') having R_F less than that of raffinose; the formation of a second upper spot having even smaller R_F was observed when extracts were incubated with high

Table 1. *Effect of inorganic phosphate on the course of sucrose breakdown by a mould extract*

(0·5 ml. 30·0% (w/v) sucrose, 0·5 ml. water, 0·6 ml. 0·03M-sodium veronal buffer (pH 7·0) and 0·4 ml. extract of crushed *P. spinulosum* mycelium were incubated at room temperature; samples taken at intervals were analysed by paper chromatography. 0·5 ml. 30·0% sucrose, 0·6 ml. M-phosphate (pH 7·0), 0·5 ml. M-NaF solution and 0·4 ml. of the same mould extract were incubated under the same conditions and a sample was analysed after 30 min., the chromatogram being run in solvent saturated with NaH_2PO_4. Composition of the mixtures compared when the sucrose spots contained equal amounts of ketose.)

Buffer	Time (min.)	Composition of solution (mg. ketose/ml.) Upper spots	Sucrose	Fructose	Recovery of fructose (%)
Veronal	25	7·6	27·6	2·0	95
Phosphate	30	7·8	27·6	1·8	95

concentrations (10–15%) of raffinose. In addition to these substances a reducing sugar appeared in the sucrose position of chromatograms; its formation was accelerated by the addition of fructose to the reaction mixture and it was probably the same as the spot, considered to be a fructose disaccharide, which appeared when sucrose was used as substrate.

Hydrolysis of the sugars (with yeast invertase) after separation by the first development of a two-dimensional chromatogram showed that the disaccharide spot was composed entirely of fructose; the 'first upper spot' was hydrolysed to a mixture of fructose and melibiose.

Prolonged incubation with mould extracts resulted in the disappearance of fructose-containing oligosaccharides, the disaccharide remaining longer than the other sugars.

Extracts containing appreciable amounts of melibiase hydrolysed raffinose completely, but traces of melibiose could usually be detected in the reaction mixture, indicating that the liberation of fructose by these extracts was still independent of galactosidase activity.

Addition of methanol (10%, v/v) completely inactivated the melibiase. At the same time, fructosidase activity, although diminished, resulted in the formation of a non-reducing fructose-containing substance having the same R_F value as methyl β-fructofuranoside.

Stachyose. Incubation of stachyose at pH 5·0 with the extracts examined (*P. spinulosum*, *A. oryzae*, *A. niger* and Taka diastase) resulted in a slow liberation of fructose and the appearance of manninotriose. Subsequently, manninotriose was hydrolysed to a mixture of glucose and galactose; a reducing oligosaccharide having the same R_F as melibiose appeared as an intermediate in the reaction. Relatively small amounts of a reducing, fructose-containing sugar having R_F less than that of stachyose were also produced.

Addition of fructose to reaction mixtures resulted in the formation of a reducing sugar which ran in the sucrose position of chromatograms. This substance was probably the same as that considered to be a fructose disaccharide, which was formed when sucrose or raffinose was used as substrate; it gave the same yellowish colour as the difructose when sprayed with benzidine, in contrast to the reddish colour of the aldose-containing sugars.

The presence of methanol (20%, v/v) suppressed the breakdown of manninotriose and decreased the rate of liberation of fructose from stachyose; at the same time, traces of a fructoside running on chromatograms in the same position as methyl β-fructofuranoside could be detected in the reaction mixture.

Electrophoresis of mould extracts in 0·01M-phosphate buffer at pH 6·8 resulted in the loss of α-galactosidase activity and the separation of a single fructosidase band. The fructosidase separated in this way from an extract of *A. oryzae* mycelium liberated fructose from sucrose, raffinose and stachyose; there was thus no indication that more than one fructosidase was involved in the breakdown of these sugars.

Melezitose. Melezitose was completely hydrolysed at pH 5·0 by the mould extracts examined (*P. spinulosum*, *A. niger*, *A. oryzae*). Electrophoresis of mould extracts in 0·01M-phosphate (pH 6·8) resulted in a slow migration of melezitase activity (i.e. ability to produce monosaccharide from melezitose) towards the anode, away from the invertase. An enzyme capable of hydrolysing turanose to a mixture of glucose and fructose, and relatively small amounts of the fructosidase were present in the same position as the melezitase. Turanase activity was not found on any other region of the paper strip, but the fructosidase probably represented small amounts of invertase which had remained adherent to the paper at the base-line; the main invertase band did not attack melezitose. Incubation of melezitose with sections of paper carrying the separated melezitase resulted in liberation of glucose and fructose; a fructose-containing spot in the disaccharide position of the chromatogram appeared as an intermediate. The disaccharide was present only in small amounts and, because of difficulty in distinguishing between sucrose and turanose on chromatograms, its nature was not established.

An extract of *A. niger* in acetate buffer (pH 5·0) which had been passed through a column of aluminium hydroxide (British Drug Houses Ltd., 'Dry') was incapable of attacking either melezitose or turanose, although invertase activity was not greatly diminished.

Attempts to confirm that the mould fructosidase was concerned in the breakdown of melezitose by extracts of *A. oryzae* or *A. niger* were not successful: it was not found possible to demonstrate the transference of fructose residues to added fructose or to methanol (20%, w/v).

Maltose. The breakdown of maltose by mould extracts at pH 7·0 resulted in the liberation of glucose, and the formation of two other reducing oligosaccharides having R_F values less than that of maltose. Of these sugars, one was possibly 6-α-glucosyl-D-glucose (isomaltose) and the other 4-α-isomaltosyl-D-glucose ('panose'), formed from maltose by the action of an α-glucosidase from *A. niger* (Pan, Andreasen & Kolachov, 1950; French, 1951) and *A. oryzae* (Pazur & French, 1952).

Further investigation of the course of maltose breakdown was not attempted because maltase activity separated by electrophoresis of mould extracts did not attack sucrose.

Methyl α-glucoside. The hydrolysis of methyl α-glucoside at pH 5·0 resulted from the action of a mould enzyme which could not be detected after electrophoresis of the extract, and therefore appeared to be distinct from the maltase.

The majority of the extracts examined contained relatively small amounts of methyl α-glucosidase, although greater amounts were usually present in mycelia grown on glucose media. Glucose was the only detected product of the hydrolysis of methyl α-glucoside.

Trehalose. Mould extracts hydrolysed trehalose at pH 5·0; in this case, also, glucose was the only observed product of the reaction. Comparison of the various 'α-glucosidase' activities of extracts of mycelia grown in different sugars showed that maltose activity was greatest in extracts of material grown on maltose or raffinose media; methyl α-glucosidase in an extract of material from a glucose medium, and trehalase in extracts of mycelia from maltose and sorbose media.

DISCUSSION

The identification of mould invertase with an α-glucosidase ('glucosaccharase') was suggested originally by Kuhn (1923) in order to account for the inhibitory action of α-glucose, presumed to result from competitive adsorption of the sugar on the enzymic surface. In contrast, yeast invertase was inhibited by fructose rather than by α-glucose and was considered to be a fructosidase.

Subsequent reports (Kuhn & Münch, 1927) that mould invertase preparations attacked melezitose but not raffinose provided support for the glucosaccharase theory, since it was not expected that the enzyme would attack a substituted α-glucosidic residue.

However, later workers found that mould extracts commonly, if not always, contained a fructosidase, usually demonstrated by the ability of such preparations to attack raffinose under conditions where melibiose was not attacked (Hofmann, 1934; Hestrin, 1940). The preparation used by Hestrin failed to attack melezitose, and therefore could not contain a glucosaccharase, but in other cases it was assumed that both glucosaccharase and fructosidase were present. Despite these observations, the idea that mould invertase is typically an α-glucosidase has been restated in recent reviews (Neuberg & Mandl, 1950; Gottschalk, 1950).

In previous publications (Bealing & Bacon, 1951, 1953) it was reported that mould extracts contained a transfructosidase. This was also discovered independently by Wallenfels (1951) and Pazur (1952). It was shown that the action of this enzyme on sucrose resembled that of the mould invertase described by earlier authors, i.e. it possessed properties hitherto attributed to a glucosaccharase, thus raising the question whether the latter enzyme did, in fact, exist.

The experiments described here go further than those previously reported (e.g. by Hestrin) in showing (*a*) that the inhibitory effects of glucose on the breakdown of sucrose by extracts of *P. spinulosum* are manifested not only in a decreased rate of change of optical rotation, but also in an apparent diminution of fructosidase activity, and (*b*) that these extracts attack raffinose, stachyose, and melezitose in a manner which can be explained by the action of the fructosidase and of other glycosidases (melibiase, turanase) known to be present.

The action of mould extracts on sucrose leads to the formation of fructose-containing oligosaccharides ('upper spots') by the transference of fructose residues to sucrose (Bealing & Bacon, 1953). The inhibitory action of glucose, which is exerted mainly on oligosaccharide formation, could thus be explained by the supposition that this sugar also acts as an acceptor for fructose, in competition with sucrose.

$$\text{Sucrose} + \text{enzyme} \rightarrow \text{Fructose} - \text{enzyme} + \text{glucose}, \quad (1)$$

$$\text{Fructose} - \text{enzyme} + \text{glucose} \rightarrow \text{Fructose} - \text{glucose} + \text{enzyme}. \quad (2)$$

If the product (fructose – glucose) of reaction (2) were sucrose the net effect would be a slower rate of progress of reaction (1).

Under these circumstances, free radioactive glucose should become incorporated in sucrose. This has been demonstrated by Edelman & Bealing (1953), the rate of incorporation being much greater than that required to account for the inhibitory action of added glucose and indicating the existence of a continuous interchange of glucose between the free state and combination in sucrose. The fact that sucrose, rather than a β-glucoside, is formed in the reaction is in accordance with the observations of earlier workers that mould invertase is inhibited by α-glucose rather than by the β-form.

The inhibitory action of glucose on the rate of sucrose breakdown may therefore be attributed to its participation in a reaction catalysed by mould transfructosidase, and it becomes unnecessary to presume the existence of a glucosaccharase to account for the effect.

The inhibitory action of fructose resembles that of glucose in that oligosaccharide formation is decreased, but differs in giving rise to a new substance, apparently a reducing disaccharide composed of two fructose residues. A substance of this type might be expected to appear if fructose, like glucose, inhibits the reaction by functioning as an alternative acceptor for fructose residues.

The inhibitory actions of other sugars seem to be explicable in similar terms; whether any 'true' competitive inhibition occurs cannot be decided with existing information. With alcohols it seems possible that there is some inactivation of the enzyme distinct from the effect of the transfer reaction whereby alkyl fructosides are produced.

The experiments with raffinose and stachyose as substrates confirmed the presence of a fructosidase in all the extracts examined. The liberation of fructose was accompanied by fructose transfer, thus strengthening the tentative conclusion of Bealing & Bacon (1953) that the transfructosidase and fructosidase (invertase) activities are due to the same enzyme.

All the enzyme preparations attacking melezitose could also attack turanose, suggesting that the first stage in the hydrolysis of the trisaccharide is the formation of glucose and sucrose, the latter subsequently being acted upon by the fructosidase. However, the turanase was not obtained entirely free from invertase activity, and it was not possible to identify the disaccharide formed as an intermediate when the partly purified enzyme acted on melezitose. It might therefore be argued that the slight invertase activity of turanase preparations was an indication that turanase itself (an α-glucosidase) is capable of attacking sucrose, but this is made less likely by the fact that the course of the action on sucrose resembled qualitatively the action of the transfructosidase.

The variations in maltase, methyl α-glucosidase, and trehalase activities when the mould was cultured on different sugars suggest strongly that these are three distinct enzymes, confirming the observation of Hestrin (1940) and at the same time refuting the theory of Weidenhagen (1932). The hypothetical glucosaccharase could be one of these, or distinct from all three.

The maltase was separable by filter-paper electrophoresis, and had no action on sucrose. This confirms the results of Hestrin (1940), who inactivated the invertase, but not the maltase, of Taka diastase by treatment with heat or acid. Methyl α-glucosidase was lost during electrophoresis, but the effect of this loss on the total invertase activity was not investigated.

There are therefore no grounds for postulating the existence of a glucosaccharase in extracts of *P. spinulosum*, although the general properties of the invertase present are those of the mould invertases previously described. Although it is not excluded that a glucosaccharase may occur in other moulds it would seem better to abandon the use of the term until some positive evidence for its existence is produced. Some unpublished experiments of ours suggest that the invertase extracted from the viscera of bees acts as a transfructosidase and attacks raffinose without the preliminary action of a melibiase. A detailed investigation of this and other enzymes hitherto classified as glucosaccharases seems desirable.

SUMMARY

1. The inhibition by free sugars and alcohols of sucrose breakdown by extracts of *Penicillium spinulosum* has been studied by paper chromatography.

2. The rate of formation of oligosaccharides (transfructosidation) from sucrose was decreased by the addition of glucose or fructose. In the presence of the latter a reducing fructose disaccharide was formed.

3. In the presence of various alcohols the breakdown of sucrose was accompanied by the appearance of substances presumed to be the corresponding alkyl β-fructofuranosides.

4. No evidence could be obtained for the participation of inorganic phosphate in the action of the fructosidase.

5. The action of extracts of *P. spinulosum* and other moulds on raffinose and stachyose could be explained by the presence of melibiase and of a fructosidase possessing transfructosidase activity.

6. The mould extracts examined hydrolysed melezitose completely, but fractionation by adsorption on aluminium hydroxide, or by filter-paper electrophoresis, yielded preparations inactive towards melezitose, but still possessing the characteristic transfructosidase activity towards sucrose. All preparations capable of attacking melezitose could also attack turanose.

7. None of the observations made requires the postulation of an α-glucosidase capable of attacking sucrose or sucrose derivatives. It is therefore suggested that the concept of a 'glucosaccharase' (Kuhn, 1923) should be abandoned until positive evidence for the existence of the enzyme has been obtained.

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Separation of Uroporphyrin Esters I and III by Paper Chromatography

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The separation of free porphyrins according to the number of carboxyl groups on their substituent side chains has been achieved by paper chromatography (Nicholas & Rimington, 1949, 1951*a*). This method is very sensitive, and has already revealed the presence of hitherto unknown porphyrins in pathological urines (McSwiney, Nicholas & Prunty, 1950; Nicholas & Rimington, 1951*a*; Rimington & Miles, 1951); it does not, however, separate the position isomers coproporphyrins I and III or uroporphyrins I and III. The method of Chu, Green & Chu (1951), in which the porphyrin esters are used, allows the identification of protoporphyrin, coproporphyrin I, coproporphyrin III and uroporphyrin I in mixtures. But these workers did not study uroporphyrin III and we have found that the uroporphyrin isomers do not separate under their conditions.

The identification of a uroporphyrin isolated from natural sources as the series I or series III isomer has relied in the past mainly on comparison of the melting point of the esterified specimen with the melting points of the supposedly pure isomers. Only since the isolation by Nicholas & Rimington (1951*b*) of unequivocal uroporphyrin III from turacin, has the melting point (264°) of pure uroporphyrin III ester been established beyond doubt. Even so, reliable estimates of the composition of a specimen from natural sources cannot be made simply from melting-point comparisons, because of the unexpected form of the mixed melting-point curve recently established in this laboratory for uroporphyrin esters I and III (Nicholas & Rimington, 1953). A powerful tool has been provided, however, by the use of this curve coupled with the specific decarboxylation of uroporphyrin mixtures to the corresponding coproporphyrins, with subsequent determination of the proportions of the latter (cf. Rimington & Miles, 1951).

In this paper we describe a paper-chromatographic method which gives good separation of the uroporphyrin esters I and III. By a combination of this method with that of Chu *et al.* (1951) all five main natural porphyrins (uroporphyrins I and III,

coproporphyrins I and III and protoporphyrin) can be separated on a single paper square (see preliminary note, Falk & Benson, 1953).

Some results of the application of the method to the analysis of natural uroporphyrin mixtures are described, the significance of the results being discussed more fully by Nicholas & Rimington (1953).

EXPERIMENTAL

Materials

Solvents. The kerosene was commercial heating paraffin, distilled over $CaCl_2$; the fraction boiling at 190–250° was used. Chloroform, B.P., was washed three times with water and dried over $CaCl_2$. Ethanolic chloroform: to chloroform treated as above, ethanol was added to a concentration of 1% (v/v). Dioxan was purified by the method of Eigenberger (1931). *n*-Propanol was redistilled and had b.p. 95–97°. Melting points were corrected.

Specimens from pathological sources. Sample 4 (Table 1) was collected from band *Ci* of Fig. 1 of Rimington & Miles (1951); sample 2 was material *B* of Table 1 of the paper of Rimington & Sveinsson (1950). Samples 5–9 were all 'Waldenström' esters prepared from the urine of cases of acute porphyria in relapse, as described by Nicholas & Rimington (1953). The isolation from a case of porphyria cutanea tarda of sample 10 was described by Macgregor, Nicholas & Rimington (1952). Sample 11 was isolated from the urine of the same patient during a different attack.

Methods

Chromatography was by the ascending method in closed glass jars, at 22–23°. The solvent to saturate the atmosphere was put in the bottom of the jar and the developing-solvent mixtures in a Petri dish in which the paper square, rolled into a cylinder, was stood. 20 cm. squares of Whatman no. 1 paper were used, the porphyrin esters being applied from freshly prepared solutions in chloroform as spots along a base-line 2 cm. from one edge. The positions of spots after development were determined by their fluorescence in ultraviolet light.

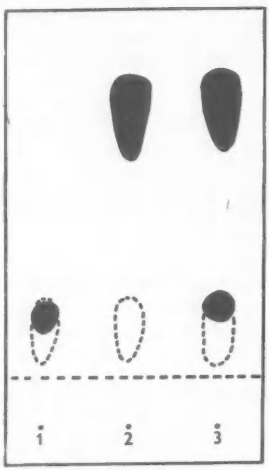

Fig. 1.

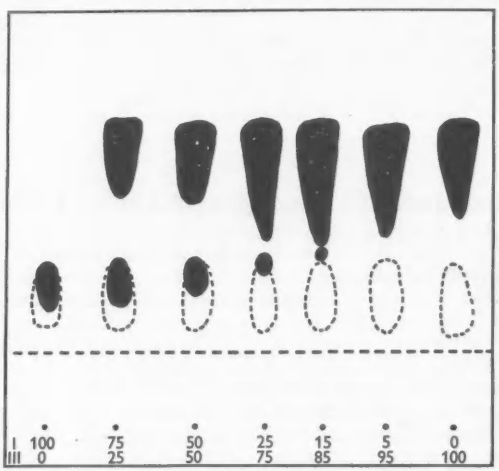

Fig. 2.

Fig. 1. Paper chromatogram obtained using method A. (1) Uroporphyrin I, (2) uroporphyrin III, (3) equal quantities of uroporphyrins I and III. Broken lines show positions of spots before final development. (For details of method, see text.)

Fig. 2. Paper chromatogram obtained by application of method A to a series of mixtures of pure uroporphyrin esters I and III. (For details of method, see text.)

Standard reference substances. Uroporphyrin I ester, m.p. 293° (Table 1, sample 3) was fraction *Ai* shown in Fig. 1 of the paper by Rimington & Miles (1951); further data concerning it are given by Rimington & Sveinsson (1950). Uroporphyrin III ester, m.p. 264° (sample 1), was prepared from turacin by Nicholas & Rimington (1951*b*). Coproporphyrin I ester, m.p. 250–251°, was isolated from calf meconium and coproporphyrin III ester, m.p. 160/178° from *Corynebacterium diphtheriae*. Protoporphyrin IX ester, m.p. 230°, was prepared from haemin by the method of Grinstein (1947).

A. *Chromatography of methyl esters of uroporphyrins* (Figs. 1 and 2)

(1) Kerosene-ethanolic chloroform (see Materials section) development (atmosphere, chloroform). The paper was developed by a mixture of kerosene (4 ml.)

and ethanolic chloroform (6 ml.), until the porphyrin spots had moved about a third of the way up the paper. This was then removed and dried. Uroporphyrin esters I and III and mixtures of them move at the same rate, and no separation is achieved in this process, which merely serves to move the porphyrin esters away from the base-line and from traces of free porphyrins or other non-mobile impurities. The base of the paper was now cut off 0·5 in. below the spots to eliminate non-mobile material and to save time in the next development.

(2) Kerosene-dioxan development (atmosphere, dioxan). The cut-down square was developed in the same direction as before, with a mixture of kerosene 4 ml. and dioxan 1·5 ml., the solvent front being allowed to reach the top of the paper.

With different batches of dioxan, in spite of the most careful purification and fractionation, it was necessary to determine experimentally the proportion of dioxan to kerosene for optimal separation of the isomers.

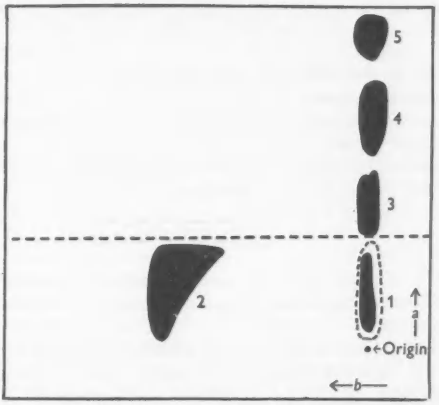

Fig. 3. Paper chromatogram obtained using method B. (1) Uroporphyrin I, (2) uroporphyrin III, (3) coproporphyrin I, (4) coproporphyrin III, (5) protoporphyrin. Developed in direction (*a*) after Chu *et al.* (1951) and the lower portion then developed in direction (*b*) with kerosene-dioxan. (For details of method, see text.)

B. *Chromatography of mixtures of uroporphyrins, coproporphyrins and protoporphyrin* (Fig. 3)

The above method was combined with the method of Chu *et al.* (1951) to analyse mixtures of the esters of the uroporphyrin isomers, the coproporphyrin isomers and protoporphyrin on a single paper square. After development by the method of Chu, the uroporphyrins remained together near the base-line. The paper was cut off parallel with the base-line, just above the uroporphyrins, leaving the separated coproporphyrins I and III and protoporphyrin on the top portion of the paper. The lower portion was then developed with kerosene-dioxan as above, but in a direction at right angles to the direction of the previous developments.

The method of Chu *et al.* (1951) involves development with kerosene-chloroform (without addition of ethanol) followed by a kerosene-propanol development. Since the uroporphyrins are moved just clear of the base-line in this process, it was not necessary to include the kerosene-ethanolic chloroform development, though this can be done if desired.

RESULTS

Uroporphyrin esters I and III move in the above processes with R_F values of about 0·02 and 0·5 respectively, the ratios being calculated from the positions of the spots before and after the last (kerosene-dioxan) development. Uroporphyrin I forms a relatively small, round, spot, very strongly fluorescent, while the uroporphyrin III spot is elongated and rather fan-shaped (Fig. 1); since the material occupies more space on the paper the fluorescence is less intense. In mixtures of the pure isomers, a 15% admixture of either was easily distinguished (Fig. 2).

Table 1 compares the chromatographic assessment of the proportions of isomers in uroporphyrin mixtures from pathological sources with that deduced from the melting point of the mixture.

DISCUSSION

The provision of a paper-chromatographic method for the separation of the isomeric uroporphyrins I and III is a considerable help in the study of the porphyrins excreted by patients with porphyria.

Uroporphyrins I and III have not yet been synthesized, and thus for the standard markers it was necessary to use carefully purified materials from natural sources. The constitution of these pigments had been established by part-decarboxylation and characterization of the resulting coproporphyrins.

It was found better to apply the materials to the paper in solution in newly washed and dried chloroform than in acetone solution. If acetone is used (cf. Chu *et al.* 1951) it is important that the solutions be freshly prepared. Decomposition sufficient to show on the chromatograms may occur within 24 hr. and becomes spectroscopically obvious after a few days.

From the paper chromatograms it is possible to make a rough estimate of the proportions of the isomers present in a natural mixture. The limit of sensitivity is about 10%; a 15% admixture of either isomer is clearly distinguishable.

Application of the method to a number of 'Waldenström' esters, the esterified uroporphyrin

Table 1. *Composition of naturally occurring uroporphyrins*

Sample	Source of material*	M.p. (corr.) (°)	Interpretation of paper chromatograms	Composition deduced from position of melting point on melting-point curves: Uroporphyrin curve†	Coproporphyrin curve‡
1	Turacin	264	III only	III only	—
			Congenital porphyria		
2	Bone	293	I only	I only	—
3	Lev.	293	I only	I only	—
4	Lev.	244	I ≧ III	—	—
			Acute porphyria		
5	Beres.	253–4	25% I	15–20% I	20–25% I
6	Salisb.	255–7	20% I	15–20% I	20% I
7	Glasg.	260	Trace I	15% I	15% I
8	Evan.	245–58	Little I	18–36% I	—
9	McLaugh.	257–8	10% I	15–20% I	20–23% I
			Porphyria cutanea tarda		
10	L. Walk.	259–61	III only	5% I	—
11	L. Walk.	256–60	25% I	14–24% I	20–25% I

* Abbreviations apply to patients' names; these are included for ease of cross-reference to the accompanying papers (Nicholas & Rimington, 1953; Kennard & Rimington, 1953).

† Cf. Nicholas & Rimington (1953).

‡ Coproporphyrin from decarboxylation of corresponding uroporphyrin sample; composition by comparison with the melting-point curve of Jope & O'Brien (1945).

fraction from acute porphyria urines, showed the presence of both isomers, with uroporphyrin III always predominating in this type of porphyria (samples 5–9). In the case of porphyria cutanea tarda studied, the III isomer was again present in the higher proportion (samples 10 and 11), while in the congenital porphyrias (samples 2–4) mainly uroporphyrin I was excreted.

By reference to Fig. 1 of the paper of Rimington & Miles (1951) it will be seen that fraction *A*i (sample 3 of the present paper) was the main uroporphyrin fraction, and that fraction *C*i (sample 4 here), a relatively very much smaller fraction, separated from the main band. The isomer analysis of these two fractions shows not only the predominance of uroporphyrin I in congenital porphyria but provides an interesting example of the behaviour of certain mixtures of the isomers as an entity, perhaps due to 'molecular compound' formation (cf. Nicholas & Rimington, 1953).

It is evident that the proportion of the two isomers varies somewhat between different cases of acute porphyria. The samples from a case of porphyria cutanea tarda show clearly that the proportions of the uroporphyrins excreted may vary, in this disease, from one attack to another.

SUMMARY

1. A paper-chromatographic method is described which separates the isomeric uroporphyrin esters I and III.

2. In conjunction with the method described by Chu, Green & Chu (1951), this new method allows the identification, on a single paper square, of the main natural porphyrins: uroporphyrins I and III, coproporphyrins I and III and protoporphyrin.

It is a pleasure to acknowledge the encouragement we have received from Prof. Rimington and his kindness in providing the pure samples and the natural mixtures.

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Identification and Classification of some Porphyrins on the Basis of their X-ray Diffraction Patterns

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The close structural and chemical similarity of the various porphyrins makes differentiation between them by the usual chemical and physical methods difficult. Successful characterization, using infrared spectra, has been reported by Gray, Neuberger & Sneath (1950) and by Falk & Willis (1951). X-ray diffraction patterns, being dependent on composition, molecular configuration and crystal structure, suggested an alternative method of identifying these substances. The use of X-ray diffraction in a particular instance in this field was reported by Siedel & Winkler (1943) but no numerical data were quoted. It is surprising that, while the X-ray crystallography of the related phthalocyanin pigments has been extensively studied (Robertson, 1935, 1936; Robertson & Woodward, 1937, 1940), little attention has been paid to the porphyrins, though a preliminary report on tetramethylhaematoporphyrin has been given by O'Daniel & Damaschke (1942), on aetioporphyrin I by Christ & Harker (1942) and on tetrabenztriazaporphin by Barrett, Linstead, Tuey & Robertson (1939). One reason for this neglect may be the scarcity of the materials and the difficulty of obtaining pure specimens. Chromatographic methods, recently introduced (Nicholas & Rimington, 1949, 1951*a*; Nicholas, 1951; Chu, Green & Chu, 1951), have now simplified preparative procedures and provided sensitive criteria of purity. This paper describes an investigation of the X-ray diffraction patterns of powder samples of the methyl esters of such carefully purified porphyrins.

EXPERIMENTAL

Most of the samples crystallized as fine prisms or needles of microscopic size, and attempts at growing large crystals were unsuccessful. The Debye-Scherrer powder technique was therefore used throughout. The samples (about 10 μg.) were enclosed in thin-walled capillary tubes. The photographs were taken in a cylindrical camera 6 cm. diameter with filtered copper radiation. Diffraction patterns of some of the 'Waldenström' esters and of artificially prepared mixtures were recorded on a flat plate 6 cm. from the specimen. The diameters of the arcs were measured with a Hilger film measuring rule and the intensities estimated visually. Measurements of interplanar spacing were accurate to about 3%. The quality of the photographs was greatly influenced by the degree of crystallinity of the powder, diffuse bands being frequently encountered. When possible, definition was improved by repeated crystallization.

MATERIALS

Porphyrins are most readily prepared from natural sources, but these usually contain complex mixtures of different porphyrins. Even synthetic methods lead to mixtures which are difficult to separate. All specimens used for this work were rigorously purified by chromatography of their esters, recrystallization, etc. (Nicholas & Rimington, 1949, 1951*a*; Nicholas, 1951). Their origin was as follows.

Porphyrin esters of known structure

Protoporphyrin dimethyl ester IX. (*a*) Specimens prepared from haemin (Grinstein, 1947), m.p. 225°. We are grateful to Mr J. Scott and Dr J. Kench for providing some of these specimens. (*b*) An exceptionally well crystallized specimen, m.p. 227°, from faeces of a case of porphyria cutanea tarda (Macgregor, Nicholas & Rimington, 1952).

Mesoporphyrin dimethyl ester IX. Prepared from protoporphyrin, m.p. 212°.

Mesoporphyrin dimethyl ester I. Synthetic specimen from H. Fischer's collection for which we thank Prof. S. Goldschmidt; m.p. 190°.

Deuteroporphyrin dimethyl ester IX. Prepared from haemin (Fischer & Orth, 1937), m.p. 218°.

Coproporphyrin tetramethyl ester I. (*a*) Synthetic specimen from H. Fischer's collection, rechromatographed, m.p. 256°. (*b*) From calf meconium, m.p. 250–251°.

Coproporphyrin tetramethyl ester III. (*a*) From *Corynebacterium diphtheriae* (Gray & Holt, 1948); m.p. 160/178°. (*b*) Similarly prepared and kindly provided by Prof. J. Hale; m.p. 154/179°. (*c*) Isolated from pathological materials; m.p. 154/174°. (*d*) Isolated from pathological urine, m.p. 178/183°; crystallized spontaneously in the higher melting-point form.

Uroporphyrin octamethyl ester I. From congenital porphyria urine (Rimington & Miles, 1951) rechromatographed upon $CaCO_3$, m.p. 293°.

Uroporphyrin octamethyl ester III. From turacin (Nicholas & Rimington, 1951*b*); m.p. 264°.

Porphyrin esters which are presumably mixtures or for which the structure has not been definitely established

Uroporphyrin '*Beresf.*' Ester of a 'Waldenström' porphyrin (octacarboxylic only) from a case of acute porphyria; m.p. 260/261°. Yields coproporphyrins I and III on decarboxylation. Three different preparations from this patient were examined.

Uroporphyrin '*P.Wri.*' Similar to above; m.p. 262°.

Uroporphyrin '*McLaugh.*' Similar to above; m.p. 255/256°.

Uroporphyrin '*L.Walk.*' Ester of a urinary porphyrin (octacarboxylic only) from a case of porphyria cutanea tarda; m.p. 260°. Yields coproporphyrins I and III on decarboxylation.

Uroporphyrin '*Ance.*' Ester of a urinary porphyrin (octacarboxylic only) from a case of cutaneous porphyria (Dr J. Canivet); m.p. 272–278°.

Uroporphyrin '*Turp.*' Similar to above; m.p. 272/279°.

Uroporphyrin '*Four.*' Similar to above; m.p. 260/269°.

Uroporphyrin '*McCawl.*' Ester of a urinary porphyrin (octacarboxylic only) from a case of porphyria cutanea tarda; m.p. 260°. Crystallizes in stout needles or fasciculi of fine, hair-like needles. Behaves abnormally on decarboxylation.

Uroporphyrin '*II.*' Octamethyl ester of a porphyrin prepared synthetically by Dr S. F. McDonald of Cambridge; m.p. 256–258°. Constitution still uncertain but X-ray pattern strikingly similar to uroporphyrin 'McCawl.'

Uroporphyrin mixtures. The mixtures of uroporphyrins I and III were prepared by mixing $CHCl_3$ solutions of the two esters in the required proportions, evaporation to dryness, dissolving the residue in a little benzene and inducing crystallization by the slow addition of light petroleum. The mother liquor in each case contained only a trace of porphyrin ester. The more usual solvents, $CHCl_3$: methanol, were avoided as complete crystallization could not be obtained. It was, however, observed that crystallization from $CHCl_3$: methanol was poor until the composition 75% III isomer + 25% I isomer was reached; this mixture separated readily in very well formed crystals.

RESULTS AND DISCUSSION

Measurements for the interplanar spacings from different specimens of the same porphyrin were in satisfactory agreement in every case. Only one set of results is therefore reproduced under each heading in the tables. The patterns of some of the powder photographs are shown in Fig. 1 and the calculated interplanar spacings and relative intensities in Table 1.

A comparison of the series I and III isomers showed these to possess distinctly different crystal structures, consequent upon the differences in symmetry of arrangement of side chains between the series III and I types of molecules. The difference was marked for all the porphyrin isomers compared (Table 1).

Powder photographs of mechanical mixtures show all lines, characteristic of the various components, superimposed. In mixtures of coproporphyrin esters, the presence of about 15% of either isomer could be detected; this is the order of sensitivity given by other techniques (cf. Falk & Benson, 1953). If, however, two porphyrin isomers crystallize together from the same solvent, they either form crystalline solid solutions showing a distinct X-ray powder pattern or the presence of one isomer interferes with crystallization of the

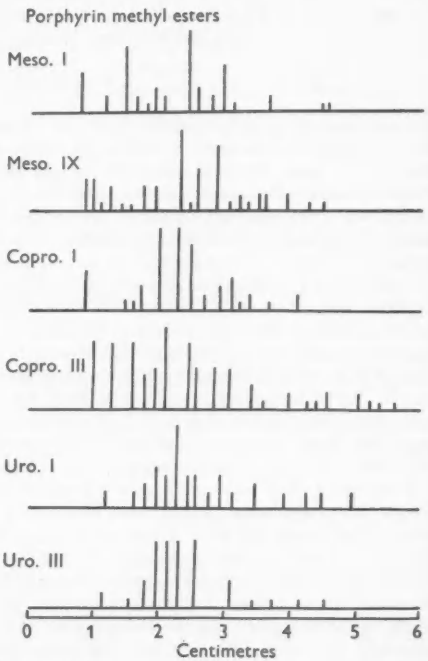


Fig. 1. Schematic representation of the positions and relative intensities of diffracted X-rays on the powder photographs of various porphyrin esters.

major constituent. In the case of uroporphyrin III, the presence of even 5% or less of the series I isomer results in a diffuse photograph showing one or two lines only. This method would seem, therefore, to provide an extremely sensitive criterion of purity.

Typical 'Waldenström' esters produced a characteristic powder pattern, with small variations in the spacing of the individual lines; Table 2 summarizes the results obtained from the various samples examined. Waldenström (1934, 1935) and Waldenström, Fink & Hoerburger (1935) and Mertens (1936, 1937) first isolated from acute porphyria urines a uroporphyrin different from uroporphyrin I; this was thought to be uroporphyrin III, since

Table 1. *X-ray diffraction patterns of porphyrin esters*

(Interplanar spacings in Ångström units.)

Coproporphyrin I	Coproporphyrin III	Uroporphyrin I	Uroporphyrin III	Protoporphyrin IX	Mesoporphyrin I	Mesoporphyrin IX	Deuteroporphyrin IX
2·32 W	1·71 W	1·93 W	2·08 W	1·88 W	2·09 W	2·12 W	1·68 W
2·60 W	1·77 W	2·12 W	2·28 W	1·98 W	2·13 W	2·22 W	1·74 M
2·77 W	1·83 W	2·23 W	2·51 W	2·12 W	2·59 W	2·41 W	1·85 W
2·91 W	1·88 W	2·42 W	2·72 W	2·43 W	3·02 W	2·62 W	1·96 W
3·02 M	2·07 W	2·73 M	3·02 M	2·63 W	3·19 S	2·70 W	2·06 M
3·20 M	2·15 W	3·02 M	3·63 S	3·04 M	3·38 W	2·82 W	2·18 W
3·51 W	2·21 W	3·21 M	4·02 S	3·45 S	3·65 M	2·94 W	2·24 W
3·74 S	2·36 W	3·41 W	4·35 S	3·66 S	3·85 S	3·09 W	2·37 W
4·10 S	2·62 W	3·69 M	4·67 S	3·99 M	4·58 W	3·29 S	2·51 W
4·67 S	2·76 M	3·87 M	5·18 M	4·17 M	4·87 M	3·65 S	2·62 M
5·41 M	3·06 S	4·13 S	5·91 W	4·82 M	5·22 W	3·83 W	2·75 W
5·83 W	3·29 S	4·46 M	8·12 W	5·61 M	5·75 W	4·10 S	3·23 S
6·42 W	3·66 S	4·80 M		6·07 M	6·37 S	4·90 M	3·47 S
	3·82 S	5·25 M		7·31 W	8·04 W	5·44 M	3·87 S
	4·44 S	5·79 W		8·19 M		6·15 W	4·04 M
	4·82 S	8·04 W		9·61 W		6·76 W	4·35 S
	5·25 M					7·76 M	4·58 W
	5·87 S					8·59 W	5·01 M
	7·31 S					9·36 M	5·47 M
	9·51 S						6·51 S
							8·59 S

Intensity scale: S = strong, M = medium, W = weak.

Table 2. *X-ray diffraction patterns of Waldenström type esters (McLaugh., Beresf. and P. Wri.) and urinary uroporphyrin esters from cases of cutaneous porphyria (L. Walk., Ance., Turp. and Four.)*

(Interplanar spacings in Ångström units.)

McLaugh.	Beresf.	P. Wri.	L. Walk.	Ance.	Turp.	Four.
3·19 W	—	—	3·16 W	—	—	—
3·63 M	3·63 M	—	3·63 M	—	—	—
—	—	—	—	3·87 W	3·89 W	—
4·21 S	4·13 S	4·17 S	4·11 S	4·10 S	4·15 S	4·17 S
—	—	—	—	4·40 W	4·44 W	—
4·67 S	4·67 S	4·67 S	4·67 S	4·77 W	4·77 W	—
5·28 M	5·22 M	—	5·22 M	5·18 W	—	—
8·35 W	—	8·76 W	8·51 W	—	—	—

Intensity scale: S = strong, M = medium, W = weak.

Table 3. *X-ray diffraction patterns of crystalline solid solutions containing varying proportions of esters of uroporphyrins I and III*

(Interplanar spacings in Ångström units.)

Percentage of isomer I								
100	95	85	80	75–30	25	10	5	0
2·73 M	—	2·82 W	2·75 W	—	—	—	—	—
3·02 M	3·07 W	3·03 W	2·99 W	—	—	—	—	3·02 M
3·21 M	—	—	—	—	3·15 M	—	—	—
3·69 M	—	—	3·69 W	—	3·61 M	—	—	3·63 S
3·87 M	3·90 W	3·85 W	—	—	—	—	—	—
—	—	—	—	—	—	—	—	4·02 S
4·13 S	4·15 S	4·19 S	4·09 S	4·15–4·19 S	4·10 S	4·08 S	4·06 S	—
—	—	—	—	—	—	—	—	4·35 S
4·46 M	4·46 W	—	—	—	—	—	—	—
—	—	—	—	—	—	4·60 M	—	—
—	—	—	—	—	—	—	—	4·67 S
4·80 M	4·80 W	4·72 W	4·82 W	—	4·70 S	—	—	—
5·25 M	—	—	—	—	5·21 W	—	—	5·18 M

Intensity scale: S = strong, M = medium, W = weak.

coproporphyrin III was identified in the products of decarboxylation. Doubt has been cast, however, upon the homogeneity of the 'Waldenström' porphyrin by Grinstein, Schwartz & Watson (1945) and Watson, Schwartz & Hawkinson (1945). These workers considered the 'Waldenström' porphyrin to be uroporphyrin I accompanied by a hepta-carboxylic porphyrin in smaller quantity. Methyl esters prepared from 'Waldenström' urinary porphyrins crystallize very readily and have fairly sharp melting points, usually lying within the range 250–260°, not far removed from the melting point (264°) of authentic uroporphyrin III (Nicholas & Rimington, 1951*b*).

All the 'Waldenström' esters used in the present work yielded both coproporphyrins I and III on decarboxylation. A series of mixed crystals, containing variable proportions of uroporphyrins I and III was therefore examined (Table 3). Decreasing the proportion of uroporphyrin I in the mixture resulted in progressively poorer crystallization; between 75 and 30% only one powder line appeared, intermediate between the spacings of the strongest planes of the pure isomers. At 25% uroporphyrin I content, there was a sudden improvement in crystallization and a well-defined diffraction pattern was produced. On decreasing the uroporphyrin I content further, crystallization was again poor and no sharp pattern was obtained until no series I isomer was present. It is of interest that the melting points of this series lie on a curve which exhibits a pronounced minimum, suggestive of a eutectic mixture, at a content of 75% uroporphyrin I (Nicholas & Rimington, 1953).

The powder pattern of the 'Waldenström' esters resembled the pattern of the solid solutions at around 25% uroporphyrin I composition. While deductions based on such few powder lines must necessarily have limited validity, the close agreement between the interplanar spacings, as well as relative intensities, lends support to the view that these 'Waldenström' esters are solid solutions of the two isomers containing around 25% of uroporphyrin I and possibly accompanied by very markedly smaller quantities of other porphyrins (see Pl. 2 and Tables 2 and 3).

The specimen 'L. Walk.' was obtained from a case of porphyria cutanea tarda and had m.p. 260°. It appeared to be quite similar to the 'Waldenström' porphyrins examined and gave an identical powder pattern. The urinary uroporphyrins from the three French cases of cutaneous porphyria were kindly placed at our disposal by Dr J. Canivet of the Saint Louis Hospital, Paris. They were selected for comparison because other methods (Nicholas & Rimington, 1953; Falk & Benson, 1953) indicated that in each the series I isomer of uroporphyrin predominated (less markedly in 'Four'). None had experienced any porphyria symptom other than photosensitization and formation of bullae. The ester melting points were 'Ance.' 272/278°, 'Turp.' 272/279° and 'Four.' 260°; thus the first two resembled the urinary uroporphyrin of congenital porphyria, while the specimen from 'Four.' had a melting point similar to that of a typical 'Waldenström' porphyrin ester. The specimens from 'Ance.' and 'Turp.' gave X-ray powder patterns indicative of a mixture of 80–95% uroporphyrin I with 5–20% of uroporphyrin III, whilst with 'Four.' only a single line at 4·17 Å appeared, as is found in mixtures of the two isomers extending from 80 to 25% uroporphyrin I (Pl. 2 and Tables 2 and 3). Other evidence (Nicholas & Rimington, 1953) supported the conclusion that the proportion of uroporphyrin I in this material is around 75–79%.

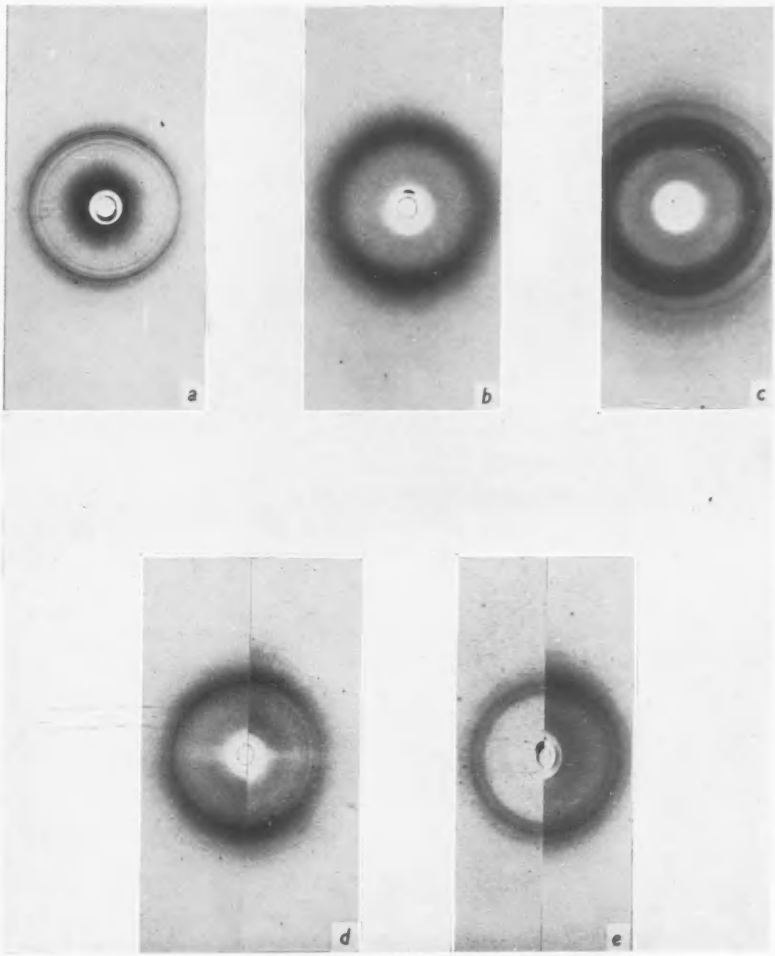
The specimen of urinary porphyrin 'McCawl.' is of special interest. This was a well crystallized methyl ester, m.p. 260°, which by elementary, methoxyl and chromatographic analyses was shown to consist of only octacarboxylic porphyrin of the uro type. The appearance of the crystals was unusual for uroporphyrin and, after melting, the mass recrystallized on the slide and remelted at the same temperature as before. The behaviour of this uroporphyrin on decarboxylation was also unusual and preliminary results have been inconclusive. The powder pattern was different from that of all the other octacarboxylic porphyrins examined except a synthetic specimen made by MacDonald (1952) (Table 4). Careful comparison showed identity with this ester. The structure of MacDonald's porphyrin is uncertain, but the synthetic route used might be expected to lead to uroporphyrin II as the main product. The infrared spectra of the synthetic and the natural porphyrins also proved to be identical (MacDonald, private communication). The elucida-

Table 4. *Comparison of X-ray diffraction patterns of esters of a synthetic uroporphyrin preparation (MacDonald, 1952) and of a urinary uroporphyrin from a case (McCawl.) of porphyria cutanea tarda*

(Interplanar spacings in Ångström units.)

Synthetic	McCawl.
2·05 W	2·21 W
2·21 W	2·57 W
2·54 W	2·90 M
2·91 W	3·08 M
3·07 W	3·29 M
3·27 W	3·47 W
3·45 W	3·59 W
3·60 W	3·82 S
3·82 M	4·15 S
4·13 S	4·46 S
4·51 S	5·31 W
	9·03 W

Intensity scale: S = strong, M = medium, W = weak.



Powder photographs of uroporphyrin methyl esters. (*a*) Pure uroporphyrin I; (*b*) uroporphyrin III containing 5% uroporphyrin I; (*c*) pure uroporphyrin III; (*d*) composite photograph; left side: crystalline solid solution containing 90% uroporphyrin I and 10% uroporphyrin III; right side, urinary uroporphyrin ester from a case of cutaneous porphyria; (*e*) composite photograph; left side, crystalline solid solution containing 25% uroporphyrin I and 75% uroporphyrin III; right side, 'Waldenström' type porphyrin from acute porphyria urine.

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tion of the structure of this material awaits the outcome of further work which is in progress.

The present work has demonstrated that powder patterns can be used to characterize porphyrins of established structure and to detect the presence of unusual porphyrins by departure from the standard patterns. Certain points of similarity between the powder patterns of various porphyrin esters are evident from Fig. 1. Full interpretation of the X-ray diffraction patterns of these substances will, however, have to wait upon the completion of studies on single crystals.

SUMMARY

1. The Debye-Scherrer powder patterns of pure porphyrin esters of known structure and configuration have been recorded.

2. A qualitative analysis of mixtures of porphyrin isomers has been made. It is shown that the powder pattern affords a very sensitive criterion of purity.

3. Powder patterns of methyl esters of porphyrins from various natural sources have been compared with recorded reference patterns.

4. The nature of the 'Waldenström' porphyrin present in urines from cases of acute porphyria is discussed in the light of the present findings.

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Studies on the 'Waldenström Porphyrin' of Acute Porphyria Urines

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Since the naturally occurring uroporphyrins have not yet been synthesized, conclusions as to their structure are based upon their chemical reactions, particularly their partial decarboxylation to the corresponding coproporphyrins. Identification of the coproporphyrin isomer indicates to which series the parent uroporphyrin belonged.

The first uroporphyrin (m.p. of octamethyl ester 290°), isolated by Fischer (1915) from the urine of a patient with congenital porphyria, yielded coproporphyrin I and was thus designated uroporphyrin I, and from supporting evidence (summarized by Nicholas & Rimington, 1951*a*) was assigned the structure porphin-1:3:5:7-tetraacetic acid-2:4:6:8-

tetrapropionic acid. No other uroporphyrin was known until Waldenström, Fink & Hoerburger (1935, see also Waldenström, 1934, 1935, 1936) and almost simultaneously Mertens (1936, 1937) reported the presence in the urine of patients with acute porphyria of a new uroporphyrin, the octamethyl ester of which melted at 258° and yielded coproporphyrin III on partial decarboxylation. It was thus regarded as uroporphyrin III, porphin-1:3:5:8-tetraacetic acid-2:4:6:7-tetrapropionic acid. The occurrence of this new uroporphyrin was speedily confirmed and porphyrin octamethyl esters, prepared subsequently from material from similar cases and all melting within the range 255–262°, were referred to generally as 'uroporphyrin III'. Confusion arose when Grinstein, Schwartz & Watson (1945) and Watson, Schwartz & Hawkinson (1945) queried the existence of the series III isomer of uroporphyrin in Waldenström porphyrins and maintained that chromatography upon calcium carbonate showed these to be associations of uroporphyrin I, the ester of which was claimed to melt at 284°, and a heptacarboxylic porphyrin belonging to the III series and characterized by a melting point of the ester of 208°. Some support for this view was forthcoming from the work of McSwiney, Nicholas & Prunty (1950) and of Gray (1950) who all detected coproporphyrin I in the products of decarboxylation of the urinary uroporphyrins studied. The uroporphyrin ester of the former workers, it may be noted, had m.p. 271/274°.

Authentic uroporphyrin III, yielding only coproporphyrin III on partial decarboxylation, was isolated from turacin by Nicholas & Rimington (1951*a*, see also Rimington, 1939). Its octamethyl ester melted at 264°. Once this reference substance had been provided, a detailed investigation of the 'Waldenström' porphyrin became possible. The X-ray crystallographic diffraction patterns given by the octamethyl esters of pure uroporphyrins I and III and of mixtures of these in known proportions have been recorded for comparison with the patterns provided by Waldenström esters (Kennard & Rimington, 1953). Similarly, an investigation has been made of the melting-point curve of mixtures of these two reference substances. Falk & Benson (1953) have elaborated a technique for separating the uroporphyrin I and III esters by paper chromatography, while Falk & Willis (1951) have measured the infrared absorption spectra of a variety of porphyrin esters including those of uroporphyrins I and III. All these ancillary techniques have been used in the study now to be recorded. Chromatography of the pure porphyrins upon solid adsorbents (Nicholas, 1951) and upon paper (Nicholas & Rimington, 1949, 1951*b*) has ensured control of homogeneity and purity at every stage.

A brief summary of the present findings has appeared elsewhere (Rimington, 1952).

MATERIALS AND METHODS

Waldenström porphyrins. These were obtained from the urines of cases of acute porphyria in relapse, i.e. patients experiencing attacks of abdominal colic and neurological disturbances or both and passing porphobilinogen in the urine, but having no history of photosensitization or cutaneous fragility resembling epidermolysis bullosa or of jaundice accompanying the attacks. These criteria are necessary to exclude cases of porphyria cutanea tarda which may suffer attacks closely resembling those of acute porphyria (see Macgregor, Nicholas & Rimington, 1952). A further point of distinction is that in porphyria cutanea tarda, during remission, the stool porphyrin is raised much above the normal level, whilst cases of acute porphyria in remission exhibit a normal faecal porphyrin output. For comparison with these 'Waldenström' porphyrins, urinary uroporphyrins were also examined, one from a case of porphyria cutanea tarda and three from cases of cutaneous porphyria (see p. 112).

Preparation of porphyrins. Since porphobilinogen is the main excretory product in acute porphyria, the urines were either heated in a boiling-water bath for 20 min. at pH 4·2 or left at room temperature for several days until conversion of porphobilinogen to uroporphyrin was complete (see Westall, 1952). Total porphyrin was then adsorbed upon talc, esterified and chromatographed (Nicholas, 1951) the purity of the octacarboxylic fractions being checked by paper chromatography using lutidine (Nicholas & Rimington, 1949, 1951*b*). Melting points were observed on an electrically heated hot stage.

Decarboxylation. This was carried out in 1 % (w/v) HCl in sealed tubes at 180–190° for 3 hr. The coproporphyrin fraction was isolated by chromatography of the esterified product.

Separation of isomers. The isomeric coproporphyrin esters were separated by the paper-chromatographic method of Chu, Green & Chu (1951) and uroporphyrin esters I and III by a similar technique due to Falk & Benson (1953). Approximate relative proportions were estimated visually from the intensity of fluorescence and area of the spots under ultraviolet light when compared with chromatograms of mixtures of the pure constituents in known proportions.

Standard reference substances. Uroporphyrin I, ester m.p. 293°, had been prepared from the urine of a patient with congenital porphyria (Rimington & Miles, 1951), further data concerning this material being given by Rimington & Sveinsson (1950). Uroporphyrin III, ester m.p. 264°, was obtained from turacin (Nicholas & Rimington, 1951*a*).

X-ray diffraction Debye-Scherrer patterns. The technique used to obtain these photographs and tables of interplanar spacings calculable from them are recorded in a separate communication (Kennard & Rimington, 1953).

RESULTS

Infrared absorption spectra

Falk & Willis (1951) record the infrared absorption spectra of the reference uroporphyrin esters I and III and also of a typical Waldenström porphyrin ester. The latter possessed characteristics suggesting a mixture of the two uroporphyrin isomers.

The melting-point curve of mixtures in known proportions of uroporphyrin esters I and III

Jope & O'Brien (1945) have shown that the melting-point curve of mixtures of coproporphyrin esters I and III has a pronounced minimum at a point corresponding to about 10% of the series I isomer plus 90% of the series III isomer. Solutions in chloroform of uroporphyrin esters I and III were mixed in known proportions, evaporated to dryness and the residue crystallized from benzene : light petroleum. The mother liquor in each case contained only a trace of porphyrin ester. These solvents were chosen, instead of chloroform : methanol mixtures, as it was important for the purpose of the investigation to obtain the whole of the material in each mixture in the solid crystalline form.

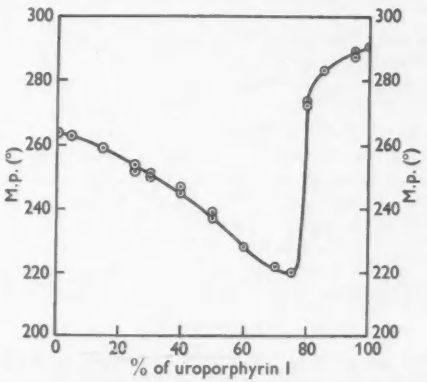

Fig. 1. Melting points of uroporphyrin esters I and III crystallized in known proportions.

The melting points of the mixtures are recorded in Fig. 1. There is a well-marked minimum at a eutectic mixture of approximately 75% I isomer and 25% III isomer. Addition of series I isomer to uroporphyrin III ester caused relatively little depression of melting point, so that a wide variation in composition is possible for mixtures melting between, say, 250 and 260°. But in the mixtures in which uroporphyrin I ester predominates, the reverse is the case. No obvious discontinuity in the curve could be detected around the region 75% series III plus 25% series I isomer.

X-ray Debye-Scherrer patterns

Kennard & Rimington (1953) report the X-ray Debye-Scherrer patterns given by the series of mixtures of known composition referred to above. They have also examined by the same technique some 'Waldenström' porphyrin esters including two used in the present study. The 'Waldenström' porphyrin esters all afforded the same pattern which was identical with that of a specimen containing 75% of the III isomer and 25% of the I isomer of uroporphyrin. This pattern is characteristic and strongly suggests the occurrence of mixed crystal formation (see Kennard & Rimington, 1953, for further discussion).

Separation of isomers by paper chromatography

Uroporphyrin esters I and III are well separated by the method of Falk & Benson (1953). The 'Waldenström' porphyrin esters examined all exhibited the same two spots, that of the series III isomer being much the stronger. In some specimens, there was so little of the isomer of series I that its spot was only just detectable. The limit of detection, from experience with mixtures of known composition, is placed at about 10%. A specimen of the original 'new uroporphyrin' (ester m.p. 258°) isolated by Mertens (1936), when examined by paper chromatography, travelled at the same rate as pure uroporphyrin ester III. It will be recalled that both Waldenström and Mertens reported that their materials gave rise to coproporphyrin III on partial decarboxylation.

Results of partial decarboxylation

The entire coproporphyrin ester fractions obtained from the 'Waldenström' porphyrin esters were examined by the paper-chromatographic method of Chu *et al.* (1951) which revealed in each case the presence of both coproporphyrins I and III. Assessment of the proportions was made (*a*) from the relative intensities and areas of the spots and (*b*) from the melting point of the crystallized ester and reference to Jope & O'Brien's curve. The findings are recorded in Table 1 which also shows the assessment made from (*c*) the melting-point curve of the uroporphyrin esters and (*d*) the relative intensities of the spots by the Falk & Benson chromatographic method of the proportions of uroporphyrins I and III in the starting material.

The agreement of these results is reasonably good and shows clearly that the proportion of uroporphyrin III is usually around 75%, as indicated also by the X-ray diffraction results.

Examination of C. J. Watson's material

The results recorded above are at variance with the conclusions of Watson *et al.* (1945). Prof. Watson kindly supplied specimens of (*a*) a 'Waldenström' porphyrin ester 'type A' (i.e. separable into components by chromatography upon calcium carbonate), (*b*) his uroporphyrin I methyl ester, and (*c*) his heptacarboxylic porphyrin ester, m.p. 208°. Chromatography of the small quantity of 'Waldenström' ester upon calcium carbonate was not successful in producing a separation; nevertheless,

Table 1. *Estimation by different methods of the composition of urinary uroporphyrins*

Specimen Designation	M.p. (°)	Proportion of uroporphyrin I from m.p. curve of uroporphyrin ester (%)	M.p. of coproporphyrin resulting from decarboxylation (°)	Proportion of uroporphyrin I from m.p. curve of coproporphyrin ester (%)	Proportion of uroporphyrin I from chromatography of specimen on paper* (%)
Beresf.[A]	253–254	15–20	204–208	20–25	ca. 20
Salisb.[A]	255–257	15–20	196–198	20	ca. 20
Glasg.[A]	260	15	180–190	15	Trace
McLaugh.[A]	257–258	15–20	200–205	20–23	ca. 10
L. Walk.[T]	256–260	15–25	202–210	20–25	ca. 25
Ance.[C]	272–278	80–82	—	—	80–95
Turp.[C]	272–279	80–82	—	—	80–90
Four.[C]	260	79 or 12	—	—	50–80

* Judged visually. Remainder uroporphyrin III in all cases.
[A] Acute porphyria.
[T] Porphyria cutanea tarda in relapse.
[C] Cutaneous porphyria. Elderly patients with no history of abdominal or nervous symptoms.

two constituents were shown to be present by paper chromatography using lutidine, the major portion corresponding with an octacarboxylic porphyrin with a small quantity of a hexacarboxylic porphyrin. Falk & Benson's method showed the former to be mainly uroporphyrin I mixed with some uroporphyrin III. When the specimen of m.p. 208° was examined separately by the lutidine method it ran consistently as a hexacarboxylic and not a heptacarboxylic porphyrin. Watson's uroporphyrin I methyl ester contained only octacarboxylic porphyrin and was mainly the I isomer, but a trace travelling as uroporphyrin III ester was detectable by paper chromatography. When this specimen was observed on the electrically heated stage, it melted at 292–293° (Prof. C. H. Gray kindly examined this material independently and found m.p. 294°), substantially the same m.p. (293°) as found by us for our pure uroporphyrin I ester, and not at 284°, the figure repeatedly given by Watson *et al.* (1945) for the melting point of the series I ester. It may be noted, however, that 294° was the melting point recorded for the uroporphyrin I ester obtained from their case 5 (Grinstein *et al.* 1945).

Examination of urinary uroporphyrins from cases of cutaneous porphyria

Dr J. Canivet of Paris kindly placed at our disposal some specimens of uroporphyrin esters prepared by him from the urines of three cases of cutaneous porphyria (for a fuller definition of this condition, see Wells & Rimington, 1953). The patients were all of middle age and had exhibited some degree of photosensitivity and of formation of bullae during the past two or three years, but not previously. They had no symptoms of colic or of disturbances of the nervous system. They did not excrete porphobilinogen, but their urines were dark and contained uroporphyrin. Teeth, etc., were normal. The esters were shown to contain octacarboxylic porphyrin only and had the following melting points: 'Four.' 260°; 'Turp.' 272–279°; 'Ance.' 272–278°. Examined by paper chromatography (Falk & Benson, 1953) and by X-ray diffraction (Kennard & Rimington, 1953), each was shown to consist of a mixture of uroporphyrins I and III. In specimens 'Turp.' and 'Ance.' the proportion of the I series isomer was 80–95%; in 'Four.' it was less than 80% but more than 50%. It is thus clear that in these cases of cutaneous porphyria the isomer of series I predominated.

DISCUSSION

The use of new techniques has made possible a much more exact analysis of porphyrin mixtures. By use of column and lutidine paper chromatography, fractions containing only octacarboxylic porphyrins may be prepared. Application to such fractions of the paper-chromatographic method of Falk & Benson (1953) and comparison of the X-ray crystallographic diffraction patterns obtained with such material with those of pure reference materials now makes possible the identification of the isomers present and allows a rough estimate of their relative proportions. Supporting evidence can be obtained from the melting point of the sample, and reference to the curve showing the melting points of mixtures in known proportions of uroporphyrins I and III, and also from the melting point and the paper-chromatographic behaviour of the coproporphyrin fraction separated from the products of partial decarboxylation of the parent uroporphyrins. All these methods, when applied to a group of typical 'Waldenström' porphyrins, demonstrate clearly that they contain predominantly uroporphyrin III accompanied by lesser amounts of uroporphyrin I. The uroporphyrin originally isolated by Mertens (1936) has been shown to behave as uroporphyrin

III, thus confirming her conclusion as to its structure. We have observed that the proportion of uroporphyrin III isomer excreted by patients with acute porphyria is somewhat variable, and on occasions the series I isomer may be detectable only as a trace, but as a rule the proportion is about 75 % uroporphyrin III to 25 % uroporphyrin I. It is of interest that it is just at this composition that X-ray crystallographic examination of artificial mixtures (Kennard & Rimington, 1953) shows a marked improvement of crystallization with the appearance of a new, characteristic pattern, strongly suggesting molecular compound formation. The ease of crystallization and recrystallization without change of melting point is a well-known property of the esters of 'Waldenström' porphyrins and is thus explained.

The urinary uroporphyrins of the cases of cutaneous porphyria are of especial interest All had uroporphyrin I as the predominant isomer. For the specimens 'Turp.' and 'Ance.' with melting points 272–279° and 272–278° respectively, no other result could have been anticipated from comparison with the melting-point curve of known mixtures of uroporphyrins I and III. The specimen 'Four.' was deliberately chosen because of its low m.p., 260°, which could indicate a mixture of either 12 % series I plus 88 % series III or 79 % series I plus 21 % series III. Examination showed conclusively that series I isomer predominated. A melting point approximately the same as that of a typical 'Waldenström' porphyrin ester is thus possible in a uroporphyrin mixture in which there is much more uroporphyrin I than III.

Our contention that uroporphyrin III is the predominant porphyrin present in typical 'Waldenström' porphyrins is supported by the present work, and some comment is necessary concerning our disagreement with Watson *et al.* (1945) who deny the existence of uroporphyrin III in such materials. We can confirm from an examination of the specimen of their material which they sent to us, that it was predominantly uroporphyrin I with a much smaller quantity of uroporphyrin III, and another substance separable on paper as a hexacarboxylic porphyrin. This we regard as an incidental impurity, similar traces of hexa- or hepta-carboxylic porphyrins having been frequently observed by us as contaminants of the uroporphyrin fraction and having required careful chromatography for their complete removal (see, for example, Rimington & Miles, 1951). Watson's ester of m.p. 208° ran similarly as a hexa- and not a hepta-carboxylic porphyrin. Prior to lutidine chromatography, we hydrolyse our esters by contact with 7 N-hydrochloric acid at room temperature for 36–48 hr. Partial decarboxylation may have occurred under these conditions, but this has never before been observed by us. Our disagreement over the melting point of uroporphyrin I ester is difficult to understand but it is readily seen from the melting-point curve (Fig. 1) that a small inclusion of the series III isomer markedly lowers the melting point.

A careful reading of the papers of the American authors leaves some doubt as to the nature of the cases they studied. No mention is made of pretreatment of the urines to convert porphobilinogen into uroporphyrin (cf. Westall, 1952), nor are full clinical descriptions of their cases given in the literature cited. We have found it difficult to separate cases of porphyria cutanea tarda in relapse from those of true acute porphyria. The former may experience colics and nervous symptoms and even excrete some porphobilinogen during their attacks (cf. Macgregor *et al.* 1952), but (*a*) their blood plasma exhibits porphyrin fluorescence during severe attacks, and (*b*) during remission their stool porphyrin is always markedly raised whilst the urinary porphyrin may be normal. Further, we have never classified as acute porphyria any case showing any degree of photosensitivity or of abnormal fragility of the skin (formation of bullae on slight trauma). The results recorded here of examination of uroporphyrins from Dr Canivet's three French cases show that in cutaneous porphyria, uroporphyrin I may predominate, even greatly, but that some such uroporphyrin mixtures may have melting points similar to those of true Waldenström esters, in the region of 260°.

SUMMARY

1. The behaviour of the methyl esters of some typical Waldenström porphyrins, obtained from carefully established cases of acute porphyria, has been compared with that of pure uroporphyrin esters I and III when examined by paper chromatography, infrared spectrophotometry, X-ray Debye-Scherrer photography and some other techniques.

2. The melting-point curve has been established for mixtures of uroporphyrin esters I and III crystallized in known proportions.

3. Deductions concerning composition from the melting point of the Waldenström porphyrin esters and of the esterified coproporphyrin fractions obtained from them by decarboxylation, also from their paper-chromatographic behaviour and X-ray powder patterns, all indicate that the Waldenström porphyrins examined contain about 75 % of uroporphyrin III and 25 % of uroporphyrin I. The X-ray examination of artificially prepared mixtures has suggested that at this composition molecular compound formation takes place.

4. Some urinary uroporphyrin esters from cases of cutaneous porphyria have been examined by similar methods. They consisted mainly of uroporphyrin I, together with smaller amounts of uroporphyrin III. One such urinary uroporphyrin had ester m.p. 260°.

5. Specimens from Prof. C. J. Watson's laboratory have been examined. The uroporphyrin methyl ester I had m.p. 292–293° and behaved as uroporphyrin I containing a trace of uroporphyrin III on paper chromatography. The 208° ester behaved chromatographically as a hexacarboxylic porphyrin.

We wish to thank Dr Falk and Miss Benson for performing the chromatographic examinations by their method, and also Prof. C. J. Watson and Dr J. Canivet for kindly placing materials at our disposal. A generous grant to one of us (C.R.) from the Trustees of the Nuffield Foundation has made possible the establishment of a Unit for the Investigation of Pyrrole Pigment Metabolism and this grant is gratefully acknowledged.

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The Protein Components of the Isolated Myofibril

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The myofibril is unique among the organized components of cells in that it requires a common substrate, adenosine triphosphate (ATP), for enzymic and mechanical activity. Both these properties survive when the myofibril is removed intact from the cell, and although it is true that the main protein component, actomyosin, has similar characteristics, extraction of this complex completely disorganizes the myofibrillar structure and results in modification of both enzymic and mechanical behaviour.

Schick & Hass (1951) investigated the solubility in salt solutions of varying ionic strength of myofibrils obtained by a procedure which involved tryptic digestion of the muscle tissue. These workers used an arbitrary method of microscopic examination to determine when solution was complete, but in view of the pronounced effect of trypsin on myosin (Gergely, 1950; Perry, 1951), and on proteins generally, it is difficult to decide to what extent the constituent proteins were modified during the preparation of the myofibrils. Information about the protein components of the myofibril other than actomyosin is scanty; for example nothing is known about the nature of the various banded features, all of which must play a part in the function of the contractile unit. Earlier electron-microscope investigations (Rosza, Szent-Györgyi & Wyckoff, 1950; Perry & Horne, 1952) have provided evidence for the washing out of some band components with no apparent modification of the underlying basic filamentous structures.

The present communication is concerned with further investigations of the properties of myofibrils prepared without the use of enzymes. In particular, the nature of the protein components extracted from the myofibril under varying ionic conditions has been investigated.

METHODS

Preparation of myofibrils. Myofibrils were prepared as previously described (Perry, 1952), using as the medium for suspension 0·078 M-borate buffer, pH 7·1 (30 ml. 0·05 M-borax and 970 ml. 0·2 M-boric acid diluted to 2·5 l.; Palitzsch, 1922). In certain cases, to reduce the amount of protein leaching out of the myofibrils, the preparation was carried out in a solution of 0·025 M-KCl and 0·039 M-borate buffer, pH 7·1. All manipulations were carried out in the cold room.

Extraction of myofibrils. Myofibrils were treated at 0° with the appropriate salt solutions so that extraction was carried out at protein concentrations ranging from 0·5 to 1·0 mg./ml. The original suspension was always in borate buffer and in consequence a low concentration of the latter was usually present during the first extraction. After a given extraction time, the myofibrillar residues were sedimented by centrifugation at 0° for 10 min. at 10000 *g*. To determine the amount of insoluble fraction, the residue was resuspended in salt solution and the extraction repeated 2–3 times. Finally total N estimations were carried out on the appropriate fractions.

Viscosity measurements. The usual procedure was to mix 5 ml. of extract with 2 ml. of 0·16 M-phosphate buffer, pH 7·0, and take 3 ml. of this solution for viscosity determination at 0° in an Ostwald viscosimeter (time of flow for water, 40–50 sec.). Total concentration of protein in the viscosimeter was 3–4 mg./ml. ATP (0·03 ml.) was added in the viscosimeter to give a final concentration of 0·0004 M.

Adenosine triphosphatase activity. The method was essentially that described earlier (Perry, 1951); glycylglycine buffer was used at pH 7·4 and glycine at pH 9·0.

Collagen estimation. Myofibrils were treated with 0·1 N-NaOH for 20 hr. at 15°, centrifuged for 10 min. at 10000 *g* and N determinations carried out on the clear supernatant and the original whole extract. Nitrogenous material not rendered soluble by this procedure was considered to consist of collagen and elastin (Lowry, Gilligan & Katersky, 1941; Robinson, 1952).

Estimations. The acid-labile P content of the myofibril and derived fractions was estimated as described in an earlier communication (Perry, 1952). Total nucleic acid (including phosphoprotein) P values were determined by the method of Schmidt & Thannhauser (1945), and N by a micro-Kjeldahl technique. Protein concentration was arbitrarily taken as 6 × nitrogen concentration.

Materials. The ATP was prepared routinely in this department by Mr E. J. Morgan. It was freed from heavy-metal impurities by treatment with 8-hydroxyquinoline, stored as the barium salt and converted to the sodium salt for use. Dr T.-C. Tsao kindly provided the electrophoretically pure, recrystallized sample of tropomyosin.

Electrophoresis. Experiments were carried out with the usual Longsworth & McInnes Tiselius apparatus, and also with the Perkin-Elmer model 38 instrument. Conductivities were determined on the protein solutions as prepared for electrophoresis.

RESULTS

Myofibril proteins soluble in 0·078 M-*borate*, pH 7·1

Freshly prepared myofibril suspensions in 0·078 M-borate buffer contained very little dissolved protein, for the addition of 10 % (w/v) trichloroacetic acid (TCA) to an equal volume of the clear supernatant obtained when the suspensions had been centrifuged for 10 min. at 10000 *g*, usually gave no detectable turbidity. During storage, at 4° in the presence of toluene, protein passed into solution with the result that after 15 days 11–12 % of the total nitrogen of the suspension had become soluble. Continued storage under sterile conditions brought little additional nitrogen into solution, and dialysis of the solution showed that it did not contain nitrogen compounds of low molecular weight. This solution of myofibrillar proteins is subsequently referred to as the soluble fraction.

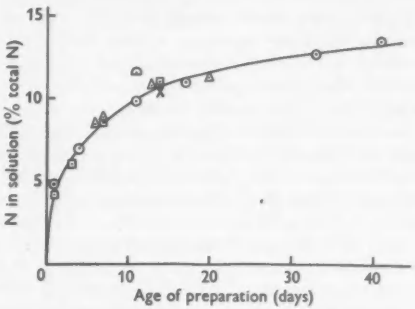

Fig. 1. Rate of solution of protein N on storing myofibril suspensions in 0·078 M-borate buffer, pH 7·1, at 4°. The results obtained with five preparations are shown, each preparation being indicated by a different set of points.

Fig. 1 illustrates the results obtained with five different myofibril preparations and shows that after a comparatively rapid solution of protein in the first day or two, the rate of extraction decreases until after 15 days it is very slow. When a suspension of myofibrils, which had been stored until 11·2 % of the total protein had gone into solution, was centrifuged down and resuspended in fresh borate buffer, the rate of solution was not increased. This indicates that the process was not a distribution of myofibrillar proteins between the gel and sol phases but rather the removal from the myofibril of one fraction more soluble in the buffer than the bulk of the myofibrillar proteins.

The soluble fraction was obtained as a clear, slightly viscous solution of protein with minimum solubility in the range pH 4·8–5·4. At 41 % saturation with ammonium sulphate little protein remained in solution, and precipitation was practically complete at 50 % saturation.

This fraction showed certain properties which suggested that it might consist, in part, of the globulin X complex. According to Weber & Meyer (1933) globulin X is precipitated at ionic strength $< 0{\cdot}005$ and there is evidence that in living muscle

only part is in solution in the sarcoplasm. Dialysis of the soluble fraction against distilled water adjusted to pH 7, or against phosphate buffers containing equimolar amounts of KH_2PO_4 and K_2HPO_4 and ranging in total ionic strength from 0·001 to 0·1, did not precipitate any significant amount of protein. On this evidence it was concluded that the soluble fraction did not contain globulin X.

Adenosine triphosphatase activity of soluble fraction. No adenosine triphosphatase (ATPase) activity was shown by the soluble fraction in the presence of calcium ions at pH's 7·4 and 9·0, nor in the presence of magnesium ions at pH 7·4. For example, when 12–14 % of the total protein had leached out of the myofibril, the whole suspension still contained powerful ATPase activity which was entirely localized in the readily centrifuged myofibrillar residue. These facts indicate that myosin was not a component of the soluble fraction.

Absence of F-actin. Precipitation of the soluble fraction with 0·01 M-acetate buffer, pH 5·1, and dissolution in a small volume of distilled water with sufficient 0·1 M-sodium bicarbonate to bring the pH back to 7, gave a very viscous solution. This was not F-actin as it did not form actomyosin on addition to a solution of freshly prepared myosin in 0·5 M-potassium chloride, i.e. ATP did not produce a drop in viscosity when added to the combined solutions.

Tropomyosin and the soluble fraction. Addition of potassium chloride to the soluble fraction, as obtained from the myofibrils by centrifugation, or to the concentrated solution obtained after isoelectric precipitation, gave a marked drop in viscosity (Fig. 2). This salt effect was reversible and strongly suggested the presence of tropomyosin, the only protein so far isolated from muscle which exhibits these properties and which is presumed to be myofibrillar in origin on the basis of the procedure employed for its extraction from washed muscle mince (Bailey, 1948).

As usually obtained, the soluble fraction contained 1·5–2·0 mg. protein/ml., and concentration was necessary before electrophoretic investigation could be undertaken. This was accomplished by suspending the solution (containing 0·05 M-phosphate buffer, pH 7·0) in a dialysis bag in front of an electric fan. When this process was carried out in the cold room at 4° the volume was reduced to one-sixth in 36 hr. without any significant amount of denaturation. Examination of this concentrated solution in the Tiselius electrophoresis apparatus revealed two main peaks (Fig. 3). These were invariably seen, but in some preparations another component, moving more slowly than the two main peaks, and present in much smaller amount, could be detected. The slower of the two main components was estimated to be present in twice the amount of the faster. Addition of a sample of recrystallized tropomyosin to the soluble fraction did not result in the appearance of a new peak but, as Fig. 3 clearly shows, the fast component increased in amount relative to the slower. This was considered as good evidence for the identity of the fast component with tropomyosin. In Table 1 are presented the mobilities of the two main components of the soluble fraction.

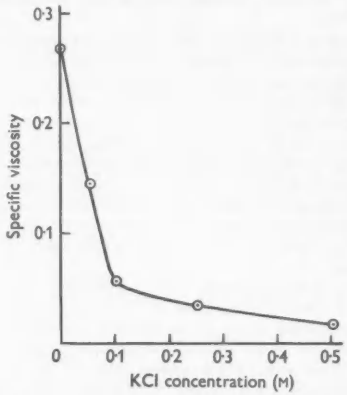

Fig. 2. Effect of KCl on the viscosity of a solution of the soluble fraction in 0·078 M-borate buffer, pH 7·1. Protein concentration approx. 0·5 mg./ml.

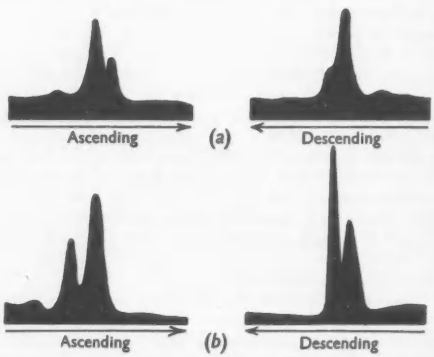

Fig. 3. Electrophoresis diagrams of the soluble fraction extracted from rabbit myofibrils, after 270 min. (*a*) Soluble fraction; (*b*) soluble fraction with added tropomyosin. Composition of buffer: 0·087 M-phosphate, pH 7·1; 0·25 M-NaCl.

On addition of potassium chloride to a final concentration of 0·5 M the viscosity of the soluble fraction dropped to a very low level (Fig. 2), and in view of the electrophoretic evidence it was assumed that at low salt concentrations the viscosity of the extract was almost entirely due to the tropomyosin present and that the main component contributed

little. On this assumption a value for the tropomyosin concentration of the soluble fraction was obtained by comparing the reduction of viscosity obtained on addition of 0·5 M-potassium chloride with that produced under identical conditions with a sample of pure tropomyosin. Table 2, showing the results of such determinations on several preparations, indicates that about 30 % of the soluble fraction consists of tropomyosin. This proportion stayed fairly constant during the extraction of the soluble fraction, and the maximum amount of tropomyosin extracted represented 4·0 % of the total proteins of the myofibril. On the basis of his organic solvent extraction method Bailey (1948) estimated tropomyosin to account for 2·6 % of the total proteins of rabbit muscle, i.e. 4·0 % of the myofibril if the latter contains 65 % of the total protein of muscle.

Table 1. *Electrophoretic mobilities of components of the soluble fraction of rabbit myofibrils*

(Electrophoresis carried out at 0° in 0·05 M-sodium phosphate buffer, pH 6·5, and 0·22 M-KCl. Values are the average of two independent determinations.)

Component	Mobility (10^{-5} sq.cm./v/sec.) Ascending value	Descending value
Fast	5·0	4·9
Slow	3·7	3·4

Table 2. *Tropomyosin extracted from myofibrils stored in* 0·078 M-*borate buffer*, pH 7·1

Prep. no.	Period of extraction (days)	Drop in relative viscosity	Protein concn. of soluble fraction (mg./ml.)	Deduced tropomyosin concn. (mg./ml.)	Tropomyosin (as % soluble fraction)
45	17	0·562	1·88	0·57	30·3
50	14	0·344	1·42	0·44	31·0
51	11	0·320	1·55	0·43	27·7

Table 3. *Nucleic-acid phosphorus content of myofibril fractions*

(All fractions were extracted with cold trichloroacetic acid and fat solvents according to the procedure of Schmidt & Thannhauser (1945). The P figure represents the total amount of ribonucleic and deoxyribonucleic acids and phosphoprotein in the fraction analysed.)

Myofibril prep. no.	Fraction	Total P (%)
45	Whole myofibrils	0·042
45	Whole myofibrils after removal of the soluble fraction	0·040
45	Soluble fraction	0·081
47	Soluble fraction	0·064
63	Soluble fraction	0·073

Phosphorus content of the soluble fraction. Hamoir (1951) has shown that tropomyosin from carp and rabbit muscle can form a crystallizable complex with nucleic acid, but it is not yet clear whether this protein exists *in situ* as such a complex or whether the nucleic acid is picked up during the extraction procedure. Consequently, it was of interest to determine the amount of nucleic-acid phosphorus in the soluble fraction, for under the conditions of extraction tropomyosin is removed from the myofibril without exposure to contamination by the nucleic acid of intracellular components such as nuclei and sarcosomes.

The soluble fraction contained variable amounts of free inorganic phosphate which presumably had leached out of the myofibril. Total nucleic-acid phosphorus values also showed some variation, the highest value obtained being 0·081 % (see Table 3). If, in the extreme case, which is rather unlikely, all this phosphorus occurred as nucleic acid associated with tropomyosin in the soluble fraction, the complex would contain 2·5–3·0 % nucleic acid. Nucleotropomyosin isolated by Hamoir (1951) from carp muscle contained considerably more nucleic acid than this.

Effect of low concentrations of potassium chloride on the extraction of the soluble fraction. To investigate the effect of low concentrations of potassium chloride on the extraction of the soluble fraction, samples of a freshly prepared myofibril suspension were left for 15 days in various solutions, the compositions of which are shown in Table 4. At the end of this period, the suspensions were centrifuged for 10 min. at 10000 *g* and a sample of each supernatant added

Table 4. *Protein passing into solution on storing myofibrils in media of low ionic strength*

Composition of medium: Borate buffer pH 7·1 (M)	KCl (M)	Turbidity on addition of TCA to the supernatant
0·039	0·20	+ + +
0·039	0·15	+ + +
0·039	0·10	+ +
0·039	0·05	+ +
0·039	0·025	+
0·039	0·01	+ +
0·039	Nil	+ + +
0·078	Nil	+ + +

to an equal volume of 10% TCA. The relative turbidities obtained, which represent approximately the relative amounts of protein which had gone into solution, are shown in Table 4. It was readily apparent that the minimum amount of protein had leached out when the myofibrils were stored in a solution containing 0·039 M-borate buffer, pH 7·1, and 0·025 M-potassium chloride.

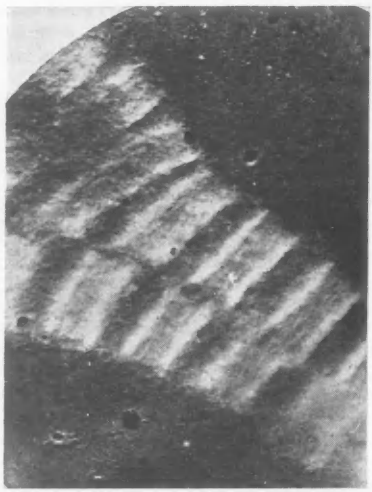

Fig. 4. Electron micrograph of rabbit myofibrils after extraction for 17 days at 4° with 0·078 M-borate buffer, pH 7·1. Gold-palladium shadowed. Magnification, × 9250.

More precise investigation indicated that only 4% of the total myofibrillar protein passed into solution during storage for 15 days in this medium. This solution was adopted for routine preparations when it was necessary to reduce the leaching out of myofibrillar protein to a minimum. It was noted that myofibrils prepared in this manner sedimented faster and formed a more compact mass in the centrifuge tube than those prepared in 0·078 M-borate, and it was more difficult to free the preparations from nuclei and other rapidly sedimenting cell components. During preparation in 0·078 M-borate the myofibrils became increasingly difficult to centrifuge down at the speeds used (Perry, 1952) and presumably in this buffer some hydration and swelling occurred.

Miscroscopic examination

Myofibrils from which the soluble fraction had been extracted were similar in general microscopic appearance to the freshly prepared material. If methylene blue was used as an aid for more careful observation, the contrast between the heavily stained bands (which frequently appeared as a double band, see below) and the lightly stained bands was greater in the stored than in the fresh preparations. This appearance of increased contrast was possibly due to the fact that, compared with the fresh material, myofibrils from which the soluble fraction had been extracted stained very lightly indeed in the less dense part of the structure.

Fig. 5. Electron micrograph of rabbit myofibrils after 30 days extraction with 0·078 M-borate buffer, pH 7·1. Gold-palladium shadowed. Magnification, × 7600.

Electron-microscope examination revealed that although there was evidence of longitudinal filamentous structures within the myofibril, fine details could no longer be recognized. A characteristic feature of these extracted myofibrils was the appearance of regular dense bands (Figs. 4, 5). Two of these bands occurred in each sarcomere, although sometimes a pair was close enough together to appear as one broader band. These bands are considered to correspond to the outer edges of the A band or perhaps to the A substance which has moved into the I band, for myofibrils isolated by the method described are usually slightly contracted (Perry, 1952). Features of the A band such as the M line and H sub-lines (Perry & Horne, 1952) were never seen in electron microscope examinations of myofibrils stored long enough to extract the soluble fraction. Further work needs to be done to decide the precise location within the myofibril of the proteins of the soluble fraction.

Double refraction of the myofibril. Examination of a number of myofibril preparations was kindly carried out with the polarizing microscope by

Table 5. *Effect of ATP on the viscosity of Weber's solution extracts of myofibrils*

Prep. no.	Age (days)	Extraction time (hr.)	Relative viscosity −ATP	Relative viscosity +ATP	Remarks
50	1	20	3·05	2·25	—
50	1	1·25	2·49	2·02	—
50	13	19	2·24	1·82	—
50	13	19	2·24	1·87	Soluble fraction removed before extraction
51	0	1	2·19	1·77	—
51	0	20	2·75	1·98	—

Dr M. Mitchinson of the Zoology Department. These investigations were of a qualitative nature but showed that all preparations had pronounced double refracting bands. No obvious diminution of the relative intensity of these bands could be seen in preparations which had been stored at 4° for 11–50 days. This suggests that the proteins of the soluble fraction are not responsible for the birefringent properties of the myofibril.

Proteins extracted by salt solutions of higher ionic strength (> 0·5)

Treatment of myofibrils with salt solutions > 0·5 M extracted considerable amounts of protein to give a viscous, sometimes slightly turbid, solution. These solutions were shown to contain actomyosin by the fall in viscosity produced on the addition of low concentrations of ATP. Myosin almost free from actin is obtained by short extractions of minced fresh whole muscle with 0·5 M-potassium chloride, whereas the isolated myofibrils produced viscous actomyosin solutions immediately they were treated with potassium chloride at this or higher concentrations. In addition, a solution containing 0·47 M-potassium chloride, 0·01 M-sodium pyrophosphate and 0·05 M-phosphate buffer, pH 6·3, such as was used by Hasselbach & Schneider (1951) to extract myosin free from actin from whole muscle, was found to extract considerable amounts of actomyosin from the myofibril in 20 min. Weber's solution (0·6 M-potassium chloride, 0·01 M-sodium carbonate, 0·04 M-sodium bicarbonate) was a very efficient agent for the extraction of actomyosin. This medium also extracted actomyosin from myofibrils which had been stored for 13 days and which had been washed free from the soluble fraction (Table 5).

When the process of salt extraction of the myofibrils was followed microscopically, cross striations were seen to disappear, although the myofibrils persisted in outline for some time, and finally the residues or ghosts which remained could be centrifuged down by high-speed centrifugation. Lithium chloride, which Bate-Smith (1937) found to be an efficient extractant for the proteins of whole muscle, brought an average of 89 % of the total myofibrillar protein into solution and less (82 %) when used in conjunction with 0·067 M-dipotassium hydrogen phosphate. Potassium chloride was less effective than lithium chloride and similarly its power of bringing myofibrillar protein into solution was reduced by 0·067 M-dipotassium hydrogen phosphate (Table 6). In general, both these salts extracted rather more protein from myofibrils which had been stored for some time under sterile conditions. Weber's solution, or salt solutions containing potassium iodide (Table 6), were the most efficient in extracting protein from the myofibril. Exhaustive extraction with these solutions reduced the insoluble residue to 2–4 % of the total nitrogen of the myofibril.

Salt extraction, other than with potassium iodide and Weber's solutions, invariably left a residue which centrifuged down as a jelly-like mass and which was shown by electron microscope examination to be composed mainly of fibrous material which could not be characterized. No myofibrils could be recognized, however, if the extraction had been exhaustive, e.g. with lithium chloride. Although potassium chloride readily extracted actomyosin, the process was by no means complete; even after fifteen extractions with M-potassium chloride the residue still showed myosin-ATPase activity. Lithium chloride was much more effective, for a short extraction with this salt brought the whole of the myosin-ATPase into solution. The residue of lithium chloride-insoluble material, averaging 11 % of the nitrogen of the whole myofibril, contained 16·7 % total nitrogen and 0·14 % total phosphorus.

The nature of this fraction is unknown, but it probably consists of components of the banded structures of the myofibril, e.g. from the A band and the Z membrane. The presence of actin in this residue seems unlikely, as lithium chloride brings into solution all the labile phosphorus, and therefore presumably all of the actin of the myofibril (Table 7). If the residue was treated with acetone and proceeded with as for the preparation of actin (Straub, 1942), none of this protein could be prepared. Attempts to prepare actin from fresh myofibrils by the Straub method were uniformly unsuccessful. It

Table 6. *Protein extracted from myofibrils by salt solutions of higher ionic strength*

Prep. no.	Time after prep. (days)	Extractant	No. of times extracted	Insoluble (%)
49	13	0·90 M-LiCl, 0·067 M-K_2HPO_4	4	17
49	21		4	16·7
50	0		4	18·9
51	0		4	18·8
51	10	0·90 M-LiCl	1 (2 hr.)	8·6
58	0		1 (3 days)	8·8
49	13		1 (20 hr.)	14·5
47	53	1·25 M-KCl, 0·067 M-K_2HPO_4	3	13·6
48	0		3	25·4
49	0		3	22·7
51	8	1·25 M-KCl	1 (1·25 hr.)	17·1
51	10		1 (2 hr.)	15·2
51	0	Weber's solution	4	2·3
62	1		1 (22 hr.)	7·4
62	1		3	4·0
58	0	0·4 M-KCl, 0·6 M-KI, 0·067 M-K_2HPO_4	4	3·1
49	4	1·25 M-KCl, 0·1 M-K_2HPO_4, 0·01 M-$Na_2P_2O_7$	4	22·3
62	6	0·47 M-KCl, 0·01 M-$Na_2P_2O_7$, 0·05 M-phosphate buffer, pH 6·23	4	24·9

Table 7. *Effect of salt extraction on the bound nucleotide of the myofibril*

(The extractions were carried out at 0° and the residue separated and washed with extractant once or twice by centrifugation for 10 min. at 10 000 g.)

Prep. no.	Age (days)	Bound nucleotide: Whole (mg. 10-min. P/ml.)	Bound nucleotide: Residue (mg. 10-min. P/ml.)	Nucleotide extracted (%)	Conditions
50	16	1·08	0·81	25	1·25 M-KCl, 1 hr.
50	16	1·08	0·16	85	0·90 M-LiCl, overnight
51	3	1·05	0·32	79	1·25 M-KCl, 2 hr.
51	3	1·05	0·09	91	0·90 M-LiCl, 2 hr.
61	3	1·43	0·29	80	1·0 M-KCl, 1 hr.
61	3	1·43	0·05	97	0·90 M-LiCl, 1 hr.
61	10	1·37	1·36	1	Soluble fraction removed and 10-min. P estimated; residue by difference
62	22	1·62	1·49	8	

appeared that most of the actin is removed by the salt extraction of the myofibrils in the first stage of the actin preparation. This is yet another example of the differences in extractability of the proteins actin and myosin in the isolated myofibril as compared with fresh minced muscle.

Collagen content of myofibril preparations. Under the conditions of estimation a very small amount of material remained insoluble, but as the nitrogen of myofibrils prepared both by the collagenase and the non-enzymic methods was rendered completely soluble by 0·1 N-sodium hydroxide the preparations were considered free from collagen and elastin.

The labile phosphorus of the myofibril

In an earlier communication (Perry, 1952) it was shown that nucleotide, mainly in the form of adenosine diphosphate, was closely associated with the myofibril and was responsible for the small acid-labile phosphorus content of these preparations. On storage of myofibril suspension in borate buffer this labile phosphorus remained constant in amount and was invariably associated with the insoluble, myofibril fraction. Table 7 shows that even after storage until 13 % of the total protein had gone into solution no significant amount of labile phosphorus could be detected in the soluble fraction. This labile phosphorus was stable in the presence of lithium chloride and potassium chloride, although the extraction of the myofibril by these salts did affect its distribution between the soluble and the insoluble fractions. Lithium chloride was very effective in extracting the bound nucleotide. Treatment for a period of 1 hr. at 0° was adequate to bring 85–100 % of the labile phosphorus into solution, whereas potassium chloride was less effective and in various experiments under similar conditions with this salt, 25–80 % was extracted.

DISCUSSION

One of the objects of the present investigation was to obtain a quantitative assessment of the various proteins present in the myofibril isolated free from sarcoplasm. Unfortunately, the fractionation methods which have been used for such studies on whole muscle, e.g. see Hasselbach & Schneider (1951), could not be satisfactorily applied to the isolated myofibril and this aim has not yet been achieved. Whereas myosin practically free from actin can be obtained from fresh whole muscle mince without much difficulty, extraction of the isolated myofibril under similar conditions produced actomyosin rich in actin.

The conventional method of actomyosin preparation involves the prolonged extraction of whole muscle and produces a very impure product. As a starting material for actomyosin preparation, isolated myofibrils have many advantages; for example, one short extraction produces an actomyosin completely free from sarcoplasmic proteins. In fact, if methods can be devised for obtaining myosin and actin independently from the isolated myofibrils, the preparation of these proteins in the purified form would be considerably simplified.

It is generally assumed that the presence of ATP in the muscle cell is necessary for the extraction of myosin free from actin. This explanation follows naturally from the behaviour of ATP with the isolated proteins, but it is not entirely satisfactory as the endogenous ATP is rapidly broken down in muscle homogenates (Bailey & Marsh, 1952). It may be that in the cell myosin can only be extracted without appreciable contamination with actin when active phosphorylation systems are working to maintain the bound nucleotide of the myofibril (Perry, 1952) in the maximally phosphorylated state, i.e. mainly as ATP. This state of affairs would be characteristic of relaxed muscle or of muscle which has been freshly minced, where the oxidative and glycolytic systems are still directed into the production of energy-rich phosphate. It would not apply to muscle which has gone into rigor or to the isolated myofibril; indeed, in the latter case, the bound nucleotide exists mainly as adenosine diphosphate. The nature of the mechanisms involved in the phosphorylation of the bound nucleotide is now under investigation.

Of the two main components which passed into solution on continued extraction of myofibrils with solutions of low ionic strength, one has unequivocally been shown to be tropomyosin. Little nucleic acid is associated with this fraction and, as it is unlikely that extraction with 0·078 M-borate buffer, pH 7·1, would dissociate a tropomyosin-nucleic acid complex, it is reasonable to conclude that tropomyosin does not occur in the myofibril as a complex containing large amounts of nucleic acid such as has been isolated by Hamoir (1951) from carp muscle. The other component which was extracted with tropomyosin has not yet been identified. It was clearly neither myosin nor the normal form of G-actin. The absence of actin from the soluble fraction was further suggested by the fact that no labile phosphorus appeared in this fraction during the extraction process. The possibility cannot be excluded that this unidentified protein was a form of inactive or denatured actin, but the fact that it was always found in constant amount relative to the tropomyosin present argues against this explanation. It may be that this component is identical with that protein invariably found in Straub's actin preparations and which has been assumed, without any clear reason, to be inactive actin.

Repeated extraction with neutral salts such as potassium chloride or lithium chloride failed to bring 9–17 % of the total myofibrillar nitrogen into solution, but if Weber's or potassium iodide solutions were used 96–98 % of the nitrogen became soluble, and indicated that the myofibril must contribute little if anything to the so-called stroma protein which accounts for 17 % of the total nitrogen of rabbit white muscle and 27 % in the red variety (Weber & Meyer, 1933; cf. Hasselbach & Schneider, 1951). The sarcoplasmic granules, or sarcosomes, which are very resistant to agents of solution and not easily removed from the muscle residue, probably contribute to the insoluble stroma fraction. In mixed rabbit muscle the sarcosomes make 3–4 % of the total muscle protein nitrogen, but in red muscle such as that obtained from pigeon breast, the figure is considerably higher (Chappell, 1952). This fact might account, in part at least, for the higher stroma values for red muscle. In rabbit white muscle the bulk of the stroma protein must be derived from insoluble nuclear residues, the sarcolemma and extracellular tissue. It is very likely that part of this stroma fraction consists of protein which has been denatured during the attainment of the degree of comminution necessary for adequate extraction. Disintegration, particularly in media of low ionic strength, can produce very appreciable denaturation of the myofibrillar components.

SUMMARY

1. On prolonged extraction of isolated rabbit-muscle myofibrils with 0·078 M-borate buffer, pH 7·1, approximately 13 % of the myofibrillar protein passed into solution.

2. This fraction soluble in 0·078 M-borate buffer contained two main components, one of which was tropomyosin, whereas the other has not yet been identified. Proteins shown not to be present in the

soluble fraction were globulin X, myosin, F-actin and G-actin.

3. The bound nucleotide of the myofibril remained associated with the myofibril on prolonged extraction with 0·078 M-borate at pH 7·1, but passed into solution on extraction with salt solutions of higher ionic strength.

4. Exhaustive extraction with potassium chloride and lithium chloride failed to dissolve 9–17 % of the myofibrillar protein, whereas extraction with Weber's and potassium iodide solutions reduced the insoluble residue to 2–4 %.

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A New Colorimetric Reagent for Carbohydrates

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Methods for the estimation of carbohydrates, and particularly polysaccharides, have been somewhat tedious till the introduction by Dreywood (1946) and Morris (1948) of the reagent anthrone in concentrated sulphuric acid. This provided a sensitive, facile and general method for carbohydrate analysis, since simple dilution of the reagent (2 volumes) by the test sample (1 volume) produces a strongly acid medium sufficiently hot to hydrolyse a polysaccharide, which then gives a colour reaction with the anthrone equivalent to that of its constituent units. Thus the reagent may be calibrated against standards of the constituent monosaccharide, since the colorimetric yield is known to be quantitative.

Unfortunately the anthrone reagent is somewhat unstable, necessitating frequent preparations, including standards for calibration. A search was therefore made for a similar but stable reagent. Various simple polyphenols, substituted phenols and aromatic amines were tried, but those which were chromogenic were also unstable in sulphuric acid. In an endeavour to stabilize resorcinol, one of the most promising reagents when used in place of anthrone, the hydroxyl groups were protected by benzoylation. When resorcinol dibenzoate in ethanolic solution and sulphuric acid were added in succession to test samples, the benzoyl groups were removed by hydrolysis with the development of a satisfactory colour, but benzoic acid crystallized out on cooling. The ditoluene-*p*-sulphonate proved resistant to hydrolysis and gave scarcely any colour. *O*-Substitution having failed, the nuclear-substituted derivative resorcinol-4:6-disulphonic acid (RDSA) was prepared, and found to be stable in aqueous solution, although insoluble in concentrated sulphuric acid. The disulphonic acid was about as sensitive a colorimetric reagent as the parent phenol, giving an intense golden-brown colour with carbohydrates. This reaction has been investigated so as to provide a reliable and simple method for carbohydrate estimation which would be useful when only very small volumes or very dilute solutions were available for analysis. Under the conditions of the reaction all the isomeric hexoses tested gave a peak at 490 mμ., but the sensitivity varied with the individual hexose (Fig. 1). For any series of determinations, therefore, the appropriate hexose must be used as standard

when calibrating the reagent. The reagent has been used chiefly for determining glucose and fructose and their polysaccharides.

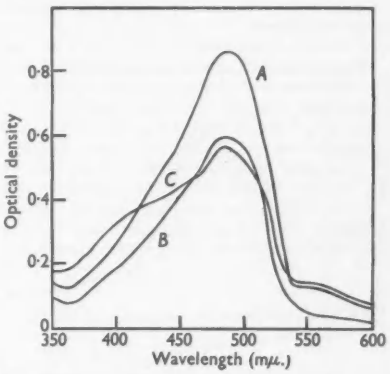

Fig. 1. Absorption spectra of reaction products of: *A*, 250 μg. glucose; *B*, 250 μg. fructose; *C*, 250 μg. galactose; each with RDSA (0·5%, w/v). Curves for sorbose and mannose are very similar to *B* and *C*, respectively.

Since somewhat different colours are given with different sugar derivatives, and since sulphonation appears to stabilize phenols in solution, other sulphonated phenols were tested, first for greater discrimination between different sugars, e.g. aldoses and ketoses, and secondly in an attempt to find a substance combining the stability of such compounds with the higher specificity and colorimetric yield of the anthrone reagent.

EXPERIMENTAL

Reagent

Resorcinol-4:6-disulphonic acid was prepared according to the procedure of Piccard & Humbert (1876) as modified by Kaufmann & de Pay (1904). Resorcinol (22 g.) was added in small portions with stirring to fuming H_2SO_4 (150 g., sp.gr. 1·875) kept at 60° in a water bath. After stirring at this temperature for 1 hr., the mixture was cooled to 0°. The calculated amount of water required to dilute the acid to 96% (13 ml.) was then added very slowly and the product which separated filtered off on to sintered glass, washed well with concentrated H_2SO_4 and drained as much as possible. The resulting paste may be used directly for the preparation of the reagent as described below, but further purification was carried out as follows: the paste (10 g.) was dissolved in 100 ml. water, and excess $CaCO_3$ added with vigorous stirring. After 30 min. the precipitated $CaSO_4$ and excess $CaCO_3$ were filtered off and washed, and the combined washings and filtrate evaporated to about 20 ml. After cooling to 0° the calcium salt together with some $CaSO_4$ was precipitated by addition of ethanol and filtered off. The precipitate was then dissolved in 20 ml. hot water and a little residual $CaSO_4$ removed by filtration. The filtrate was treated with charcoal, filtered, cooled and precipitated with ethanol as before. The product, now almost free from SO_4^{2-}, was finally recrystallized from aqueous ethanol. (Found: C, 22·6; H, 2·3; S, 19·4; Ca, 11·5. Calc. for $C_6H_4O_8S_2Ca.H_2O$. C, 22·1; H, 1·9; S, 19·6; Ca, 12·2%. Analysis by Weiler and Strauss, Oxford.) A solution of this salt may then be used as reagent. A 0·6% (w/v) aqueous solution of the Ca salt is made up (the equivalent of a 0·5% (w/v) solution of the free acid), and filtered free from dust. A solution of the free acid may also be prepared directly from the paste (1%, w/v), decolorized with charcoal and filtered, but this may contain as little as 0·5% (w/v) RDSA.

Method

The most satisfactory method is described first, followed by the studies which led up to this choice.

To 5 ml. samples containing up to 250 μg. carbohydrate in closely similar stoppered tubes, 1 ml. of 0·5% RDSA (as the Ca salt) in water is added. Conc. H_2SO_4 (A.R., 10 ml.) is added from a fast-running pipette (or burette) with shaking. After standing for 1 hr. in the dark to cool, the optical densities are read at 490 mμ., using 1 cm. cells, against the least of several blanks run concurrently, using 5 ml. distilled water. The mean value of these blanks is then subtracted from the mean of the observations.

The reagent is calibrated with standard solutions of the monosaccharide to be determined, taking every precaution to exclude dust. (All optical measurements were made on a Unicam Spectrophotometer S.P. 500.) The optical density at 490 mμ. is directly proportional to the quantity of hexose in the 5 ml. sample up to 200 μg. ($E = 0·570$), though it falls off slightly at higher densities. The concentration range 0–200 μg. is readily measured using 1 cm. cells. Starch and inulin gave results equivalent to 100% of their monosaccharide units; the more resistant polysaccharide dextran, as used clinically for transfusions, gave colours representing a 95·5% yield, the standard error of the method being ±1·0%.

It is hoped to publish further details concerning the estimation of dextran.

Variation of experimental conditions

The effect of a number of experimental conditions on the reaction was investigated.

Effect of calcium. The Ca salt behaved in exactly the same way as its equivalent of free acid. No precipitation of $CaSO_4$ was observed. A 1·2% (w/v) solution of the Ca salt gave the same intensity of colour with 120 μg. glucose as an identical solution from which the Ca had been precipitated with the calculated quantity of oxalic acid. (Optical densities 0·633 ± 0·006 and 0·638 ± 0·004, respectively.)

Concentrations of reagent and acid. The sensitivity increases with reagent concentration to a plateau at about 3% (Fig. 2), when 100 μg. glucose gives an optical density of about 0·8. This is so high that the dilution required makes it extremely difficult to eliminate scatter due to minute dust particles, and for ordinary use a 0·5% reagent solution is more satisfactory.

No variation was found when the stock conc. H_2SO_4 contained up to 3% (v/v) water.

Vessels. All reagents and apparatus must be scrupulously clean and free from dust, cotton wool strands, etc., otherwise the blanks will be appreciably coloured and the optical densities erratic. Since the validity of the optical measure-

ments rests on the quality of the blanks, it is advisable to prepare three or more blanks. The test and standard solutions were read against the lowest blank, and from these results the mean of the other blanks, which should be small (optical density against distilled water $<0{\cdot}050$ at 490 mμ.), was subtracted.

The vessels should be stoppered and closely similar in shape and thickness. Since the heat of dilution of the acid brings about reaction, the rate of cooling affects the final optical density, so that the geometric form of the vessel is important, though it does not affect the initial maximum temperature appreciably (cf. Van Slyke & Sinex, 1952). Further, the optical densities are increased to a maximum by 20 min. heating in a boiling-water bath, when the spectrum is little changed (Figs. 3, 4). If the heating is continued beyond 20 min. the optical density at 490 mμ. falls rapidly and the spectrum changes with the development of a new peak at 440 mμ. (Fig. 4). With the anthrone reaction, similar heating changes the colour visibly to grey-green.

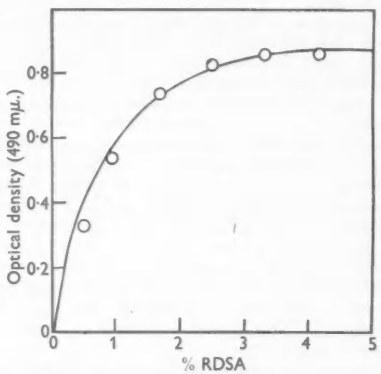

Fig. 2. Variation in optical density (490 mμ.) of RDSA/glucose reaction mixture with various concentrations of RDSA (Ca salt); 100 μg. glucose.

In these experiments B.19 Quickfit stoppered test tubes of closely similar size and weight (41 ± 1 g.) were used. There was a statistically significant difference in optical density between batches treated in light (33 g.) and heavy (41 g.) tubes (heavy $0{\cdot}400 \pm 0{\cdot}004$; light $0{\cdot}366 \pm 0{\cdot}001$; $t = 12{\cdot}22$, $n = 5$, $P < 0{\cdot}001$).

Sensitivity to light. This is greater than the experimental error. Of ten replicates, five were kept for 1 hr. in the dark, while five stood 30 cm. from a 60 w lamp. The optical densities were $0{\cdot}439 \pm 0{\cdot}004$ and $0{\cdot}411 \pm 0{\cdot}003$, respectively ($P < 0{\cdot}001$). Accordingly, all reaction mixtures should be allowed to cool in the dark, and since after the initial rapid development the colour continues to increase very slowly (approx. 1 %/hr.), the cooling should occupy a standard time (e.g. 1 hr.).

Temperature of reaction. This passes through a broad maximum when the volume of acid is approximately twice the volume of the aqueous solution. For 5 ml. of water, the addition of 8, 9, 10, 11, 12 ml. acid gave a maximum temperature of 108, 112, 118, 117·5 and 116·5°, respectively. Nevertheless, when the volume of acid added to 5 ml. of sample plus 1 ml. of reagent is increased from 10 to 12 ml. there is a decrease in optical density (corrected for dilution) of nearly 40 %. The cause of this is not apparent. The proportional decrease was identical for glucose and for dextran, i.e. the degree of reaction of dextran was not increased as compared with glucose.

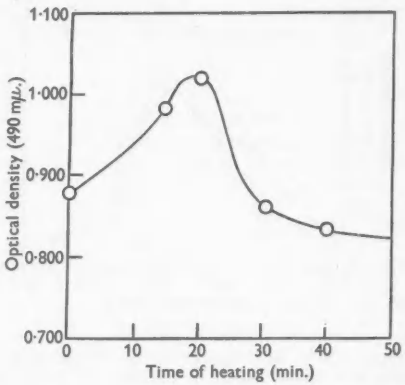

Fig. 3. Variation in optical density (490 mμ.) of RDSA (0·5 %, w/v) in glucose (250 μg.) due to heating reaction mixture at 100° for various periods.

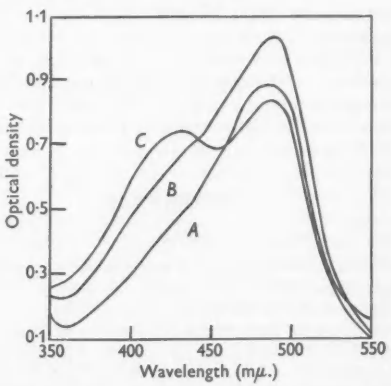

Fig. 4. Absorption spectra of RDSA/glucose reaction mixture. 250 μg. glucose, 0·5 % (w/v) RDSA. *A*, normal; *B*, after heating at 100° for 20 min.; *C*, after heating at 100° for 40 min.

Presence of chloride. Readings are increased in the presence of chlorides, as shown in Fig. 5, the shape of the absorption curve being little changed. This cannot be overcome by adding NaCl to the reagent, since crystallization occurs on cooling before the plateau of Fig. 5 is reached. The high sensitivity of the method often necessitates dilution, which normally eliminates this effect. Otherwise a correction may be made or polysaccharide material may be precipitated out with ethanol, as in the application of the anthrone method by Bloom & Wilcox (1951) to the estima-

tion of dextran in blood and urine. The sensitivity of the anthrone reagent was found to be increased (by 17%) by the presence of NaCl (1%). The acid procedure of Schaffer & Hartmann (1921) was found to give results 2% low for dextran when the hydrolysis was conducted in the presence of 1% NaCl.

Specificity of coloration. RDSA gives no reaction with the amino acids except tryptophan (pale yellow), nor with carboxylic acids, acetone, inositol, cholestan-3-one, cholest-4-en-3-one, gelatin, sodium ethylmercurithiosalicylate (British Drug Houses Ltd.) or barbitone. Various shades of yellow-brown were given by aldehydes and lactic acid and also by alcohols in much larger concentrations (probably owing to oxidation to aldehydes), and by products of sugar degradation (furfural, laevulinic acid). Ethyl acetoacetate gave a slight reaction, ninhydrin a strong claret red and dioxan a characteristic cherry red which could probably be

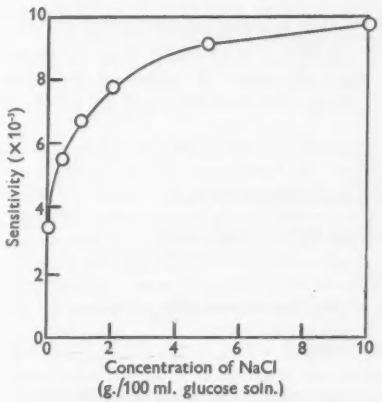

Fig. 5. Variation in sensitivity of RDSA (0·5%, w/v) with glucose due to presence of chloride. (Sensitivity = optical density (490 mμ.)/glucose present (μg.).)

used for the detection of traces of this substance. The intense golden-brown obtained with hexoses was distinguishable from the more orange colour with pentoses, and the yellow-brown colour with aldehydes (e.g. acetaldehyde, $\lambda_{max.}$ = 390 mμ.). Glucosamine gave a feeble red-brown colour only on long standing, hexuronic acids a characteristic yellow becoming black-brown, rhamnose and 6-iodofucose, red-brown, similar to the hexoses, galactal yellow-brown, 2-deoxygalactose yellow-brown and 1:2-dideoxyglucose a faint pink.

Sensitivity with various hexoses. The sensitivity to RDSA varied with the hexose as is evident from Fig. 1. The curve obtained with mannose is very similar to that shown for galactose and with sorbose to that shown for fructose. The calibration curve for fructose has a slope of about 60% of that for glucose, which is surprising since resorcinol is used in the Seliwanoff test for ketoses.

Effect of other carbohydrates. Pentoses and uronic acids in all amounts interfere with the quantitative determination of the hexoses; the reagent is more sensitive to pentoses than to hexoses. ($E_{max.}$ for 100 μg. xylose = 1770 at 470 mμ.). Glucosamine does not interfere with the determination of hexoses in the concentration range tested (up to 100 μg./100 μg. glucose).

Other reagents

N-1-*Naphthylethylenediamine.* This reagent (0·2% in conc. H_2SO_4) gave an intense crimson colour with hexoses ($\lambda_{max.}$ = 540 mμ.; optical density proportional to weight of glucose), but the reagent deteriorated as rapidly as anthrone.

Orcinol. This reagent, used similarly, showed a major peak at 435 mμ. and a lesser one at 470 mμ.; that at 435 mμ. showed a higher sensitivity than any other reagent but the calibration plot was appreciably curved. This reagent was unstable, as were the other common phenols. The relative optical densities of these compounds for 100 μg. glucose/5 ml. were as follows (all %, w/v): with anthrone 0·2% at 625 mμ., 0·25; with orcinol 1% at 470 mμ., 0·31; with resorcinol 1% at 495 mμ., 0·43; with RDSA 0·5% at 490 mμ., 0·28; RDSA 3% at 490 mμ., 0·83.

4-*Hydroxydiphenyl-4′-sulphonic acid* (Latschinow, 1873). The solution used was prepared by adding *p*-hydroxydiphenyl (twice recrystallized, 20 g.) to conc. H_2SO_4 (A.R., 200 ml.) stirred at 90° in a water bath, over a period of 30 min. After stirring for a further 30 min. at 100° all the solid had dissolved; the clear solution was cooled to 0° and cautiously added to 1800 ml. ice-water. The solution was decolorized with animal charcoal and filtered free from dust. Used as described for RDSA, the reagent gave a characteristic orange-pink colour with fructose which was absent with glucose. The fructose curve shows a sharp peak at 470 mμ., while that for the same amount of glucose shows only a low continuous absorption with no peak in the wavelength range studied (350–550 mμ.). Rhamnose and fucose gave a peak at 420 mμ. and xylose at 450 mμ. Galactose and mannose gave spectra similar to glucose. The reagent appears to be stable and calibration curves for fructose were easily reproducible.

Chromotropic acid (1:8-dihydroxynaphthalene-3:6-disulphonic acid). The recrystallized sodium salt (1% aqueous solution), used as RDSA, gave a purple coloration with glucose, yellowish red with fructose and yellow with rhamnose. It is, however, insensitive and the aqueous solution of the reagent changes colour in a few days.

Anthrone-1- and -2-sulphonic acids. These were prepared by the reduction of the corresponding anthraquinone-sulphonic acids with alkaline sodium dithionite by the method of Battegay & Hueber (1923), the sodium salts recrystallized from aqueous ethanol and were used similarly to RDSA in 1% aqueous solution. It was hoped that these might combine the higher carbohydrate specificity of anthrone with the stability of RDSA. Unfortunately, neither gave any appreciable colour with glucose or fructose under the conditions of the experiment.

Anthrone. This reagent was tested with some substances not reported by Dreywood (1946) or Morris (1948). It gave an intense scarlet colour with lactic acid, brown colours with aldehydes and more feebly with alcohols, and a reddish colour with tryptophan in large quantities: sodium alginate (polymannuronic acid) and potassium hyaluronate gave grey becoming brown and pale green respectively. Fucose, as the diacetone derivative, gave an intense blue colour.

SUMMARY

1. The use of a new colorimetric reagent, resorcinol-4:6-disulphonic acid, for the determination of hexoses and their polysaccharides, is described. The reagent has been used chiefly for the determination of glucose, fructose and their polysaccharides.

2. From 10 to 250 μg. of glucose in 5 ml. may be determined with errors of less than 1 %.

3. Inulin and starch give 100 % and dextran 95 % of the colour intensity given by their constituent monosaccharides.

We are indebted to Prof. J. R. Squire for his encouragement of this work; to various members of the Chemistry Department of this University for specimens of some of the carbohydrate derivatives tested; to Dr C. R. Ricketts for help with the manuscript, and to the Medical Research Council for studentship grants.

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Spectroscopic Studies of Carbohydrates

1. THE ACTION OF SULPHURIC ACID ON SUGARS

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The conversion by acids of pentoses to furfural and of hexoses to 5-hydroxymethylfurfural (HMF) has been extensively studied, principally by Bandow (1937), Holzman, MacAllister & Niemann (1947) and Ikawa & Niemann (1949, 1951), but little is known of the mechanism of these reactions. The subject has been recently reviewed (Newth, 1951).

Concentrated solutions of glucose in 28N-sulphuric acid blacken rapidly on heating, owing to the conversion of HMF to 'humic substances' of unknown structure, but very dilute solutions under similar conditions become rose-pink (Mendel & Bauch, 1926). This colour development was utilized by the latter authors in a method for the determination of blood glucose. The mechanism again was obscure.

It was the object of the present work to follow spectroscopically the conversion of a number of sugars to the corresponding furfural derivatives in sulphuric acid at 60°, and to note the changes in the visible spectrum that occurred when they were heated at 100°, in an attempt to throw some light on the nature of the reaction.

MATERIALS AND METHODS

Reagents. 98 % H_2SO_4, A.R. grade (sp.gr. 1·840), was used. All the sugars were the best commercial samples available, and were used without further purification, apart from being dried to constant weight over P_2O_5. Furfural was 'specially purified' (British Drug Houses Ltd.); 5-hydroxymethylfurfural was prepared according to the method of Haworth & Jones (1944), and was twice distilled at 0·1 mm. pressure, yielding almost colourless crystals, m.p. 30°.

Apparatus. Electrically heated water baths, maintained at 60° (±1°) and 100° were used for heating the samples. A pipette was calibrated to deliver 6 ml. (11·04 g.) conc. H_2SO_4 in a delivery time of 1 min. The absorption spectra of the solutions were determined with a Unicam photoelectric spectrophotometer.

Procedure. For experiments using 28·3N-H_2SO_4, 6 ml. conc. H_2SO_4 were pipetted into wide (6 × 0·75 in.) test tubes containing 2 ml. sugar solution, undue rise of temperature being avoided by swirling in ice-cold water. Blanks were prepared by mixing 6 ml. conc. H_2SO_4 with 2 ml. water under the same conditions. The tubes were placed in racks in the water bath and cooled at intervals in ice-cold water. The solutions were then poured into 1 cm. quartz cells for absorption measurements, the blank solution being placed in the compensator cell.

RESULTS

Ultraviolet spectra

Spectra in 28·3 N-sulphuric acid. The conversion of pentoses to furfural and of hexoses to HMF is not quantitative, and varies from sugar to sugar. However, the extinction due to the furfural formed from a given weight of sugar is constant (Dunstan & Gillam, 1949). The latter authors determined the yield of furfural or its derivatives from several sugars by heating 0·5–1·5 ml. of 0·1–0·5 % sugar solution at 170° with 5 ml. 85 % phosphoric acid (sp.gr. 1·689), and steam-distilling off the product. Under these conditions, considerable decomposition of HMF occurred, so that although pentoses yielded reproducible results, data from hexoses were variable and low. Ikawa & Niemann (1949) heated sugars in 79 % (w/v) sulphuric acid at 100°, and measured the absorption after 15 min. In this case, both furfural and HMF had undergone some decomposition, so values of molecular extinction coefficients (ϵ) for both pentoses and hexoses were low.

Under the present conditions, the extinction at $\lambda_{max.}$ was measured at frequent intervals during the heating of the solutions, which usually contained 5 μg. sugar/ml., and the maximum extinction noted before appreciable decomposition had occurred. As decomposition of HMF at 60° was slow, data from hexoses were as reproducible as those from pentoses. Results are illustrated in Table 1, the 328 mμ. maximum of rhamnose being due to 5-methylfurfural.

The exact position of $\lambda_{max.}$ depends on the concentration of sulphuric acid used, e.g. for glucose it is 324 mμ. in conc. acid (Bandow, 1937), and 320, 316 and about 300 mμ. in 28·3, 26·4 and 25·3 N-acid, respectively.

It was observed that sorbitol, mannitol and gluconic acid did not develop selective absorption, even after several hours, which confirms a similar observation made by Bandow (1937).

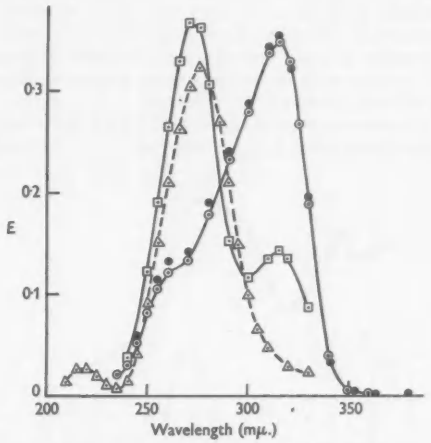

Fig. 1. Absorption spectra of decomposition products of arabinose in H_2SO_4. ⊙, D-arabinose (5 μg./ml.) after heating 25 min. at 60° in 28·3 N-H_2SO_4; ●, L-arabinose (5 μg./ml.) after heating 25 min. at 60° in 28·3 N-H_2SO_4; ⊡, D-arabinose (5·5 μg./ml.) after heating 10 min. at 60° in 37·1 N-H_2SO_4; △, D-arabinose after heating 10 min. at 60° in 37·1 N-H_2SO_4, then diluting six times with water (final concn. 5·2 μg./ml.).

Spectra in 37·1 N-*sulphuric acid.* In addition to the main absorption band at about 320 mμ., furfural and HMF in 25–37·1 N-sulphuric acid also exhibit a characteristic lesser band at about 260 mμ. Bandow (1937), using concentrated acid, observed

Table 1. *Formation of furfural or derivatives from sugars*

(28 μM solutions of sugars in 28·3 N-H_2SO_4 were heated at 60° until the ultraviolet extinction reached a maximum. The pure furfural and HMF used as references were also in 28·3 N-acid, but not heated.)

Substance	$\epsilon_{max.}$	$\lambda_{max.}$ (mμ.)	No. of determinations	Time taken to reach maximum extinction (min.)
Furfural	22 180	315	5	—
D-Xylose	21 270	315	13	10
D-Ribose*	11 430	315	5	40
D-Arabinose	11 220	315	10	25
L-Arabinose	11 220	315	5	25
D-Lyxose*	8 970	315	5	7
5-Hydroxymethylfurfural	22 750	320	4	—
D-Glucose	16 200	320	10	80
D-Galactose	13 600	320	5	50
D-Mannose	12 000	320	5	40
D-Fructose	19 550	320	8	3
L-Sorbose	14 830	320	5	3
L-Rhamnose	14 300	328	10	25

* Lyxose shows an additional maximum at 290 mμ. which exceeds that at 315 mμ. Ribose shows an inflexion at 290 mμ.

that although most sugars gave rise to such spectra, with some the relative strengths of the two bands were reversed. In the present work, no such variation was observed when 28·3 N-sulphuric acid was used, but in 37·1 N-sulphuric acid some striking changes occurred in the spectra of ribose, arabinose and lyxose. As shown for arabinose in Fig. 1, bands existed at 271 mμ. and subsidiary bands at 315 mμ. On dilution with water, the spectra showed maxima at 276 mμ. (greater) and 220 mμ.

Complete spectra of ribose in 37·1 N-sulphuric acid were plotted after 1, 2, 5, 10 and 35 min. at 60° and, although the intensity of absorption increased with time, the general picture remained the same. The minimum between the two peaks became progressively less pronounced, however, and after 35 min. the 315 mμ. maximum was little more than an inflexion.

The spectrum of glucose showed no great difference in the proportions of the two bands between the concentrations of 28–37 N-acid, neither did the spectrum of furfural alter appreciably. However, when glucose was heated for a long time in 28·3 N-acid at 100°, much of the HMF was destroyed, but the residual spectrum showed a principal maximum at 255 mμ., which exceeded E_{310} by 27 % (Fig. 2). On dilution with water, the 310 mμ. maximum shifted to about 280 mμ., but the 255 mμ. maximum shifted slightly the other way, to form an inflexion at 260 mμ.

The observation made by Holzman *et al.* (1947) that in mannose the 258 mμ. peak was the greater of the two was not confirmed here at 60° in sulphuric acid of 28·3, 31, and 37·1 N concentration, nor when the conditions of these workers were repeated, although an increase in the 258 mμ. band was seen.

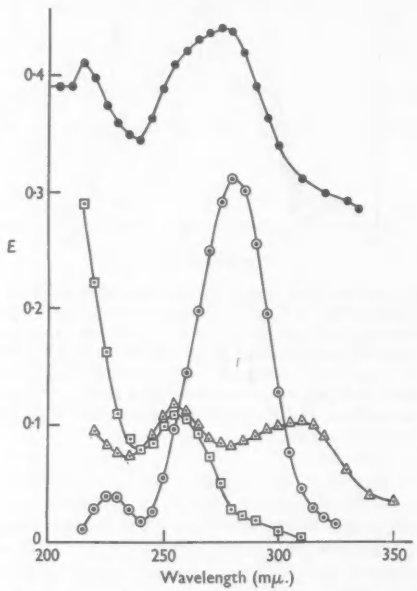

Fig. 2. Absorption spectra of decomposition products of glucose, obtained by heating in 28·3 N-H_2SO_4. ⊙, glucose heated 70 min. at 60° then diluted fifty times with water (final concn. 5 μg./ml.); ⊡, laevulinic acid (0·6 mg./ml.) in 28·3 N-H_2SO_4; △, glucose (5 μg./ml.) heated 210 min. at 100°; ●, glucose heated 200 min. at 100°, then diluted ten times with water (final concn. 25 μg./ml.).

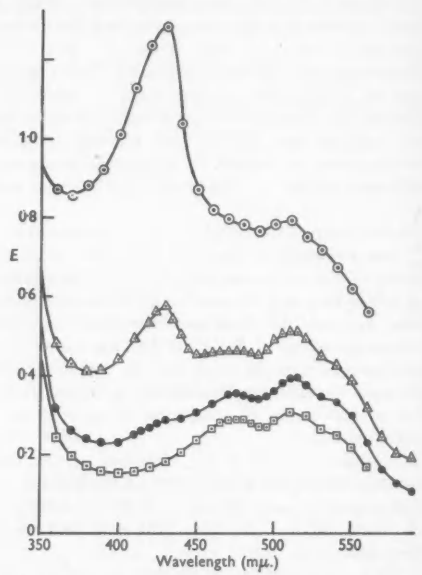

Fig. 3. Visible spectra of glucose under various conditions. ⊡, in 28·3 N-H_2SO_4 after heating for 4 min. at 100° (0·1 mg./ml.); ●, after 6·5 min. heating; △, after 10 min. heating; ⊙, after 50 min. heating.

Visible spectra

More concentrated sugar solutions were used than for ultraviolet spectra; the final mixture in 28·3 N-sulphuric acid usually contained 0·25 mg. sugar/ml. The samples were heated at 100° and cooled in ice-water at various time intervals. The spectra were plotted from about 350 to 550 mμ., and although there was often much 'ground absorption', the spectra also showed well-defined bands.

An example of such a group of spectra is shown in Fig. 3 (glucose), and spectra from other sugars are described in Table 2.

The rose-pink colour described by Mendel & Bauch (1926) was given solely by glucose. The fact

Table 2. *Description of visible spectra of sugars and related substances after heating in 28·3 N-sulphuric acid*

(Two ml. 5·6 mM-aqueous sugar solution were mixed with 6 ml. 98 % H_2SO_4 and heated at 100° for various times. Wavelengths in italics denote inflexions.)

Time of heating (min.)	2		4		8		10		15		30		35	
	$\lambda_{max.}$ (mμ.)	ε	$\lambda_{max.}$ (mμ.)	ε	$\lambda_{max.}$ (mμ.)	ε	$\lambda_{max.}$ (mμ.)	ε	$\lambda_{max.}$ (mμ.)	ε	$\lambda_{max.}$ (mμ.)	ε	$\lambda_{max.}$ (mμ.)	ε
D-Glucose	—	—	510 475	554 524	—	—	430 510	1033 921	—	—	430 510	2000 1250	—	—
D-Galactose	—	—	430 *470*	399	430	1093	—	—	430	1650	—	—	430	2215
D-Mannose	—	—	approx. 430 *470*	316	approx. 420	589	—	—	approx. 420	895	—	—	—	—
D-Fructose	—	—	470 *510*	1150	470 510	1041 941	—	—	470 515 *440*	1250 1100	—	—	470 435 515	1440 1425 1365
L-Sorbose	470	354	430 *470*	870	—	—	430 *470* *510*	1660	—	—	—	—	—	—
L-Arabinose	—	—	430	79	—	—	430	199	—	—	430 *510*	443	—	—
D-Lyxose	—	—	approx. 430	96	—	—	430	181	—	—	430 *510*	331	—	—
D-Ribose	—	—	420	138	—	—	420	294	—	—	430 *510*	501	—	—
D-Xylose	—	—	430	100	—	—	430	222	—	—	430 520	308 209	—	—
5-Hydroxymethyl furfural	470	128	470 *440* *510*	609	—	—	470 *440* *510*	1040	—	—	—	—	—	—
Furfural	—	—	465 *520*	62	465 520	92 34	—	—	465 520	172 55	—	—	465 520	346 135
L-Rhamnose	—	—	460	116	460	262	—	—	455	502	—	—	440	1440

that several sugars showed inflexions and even maxima at 510–520 mμ. suggested that the colour might be produced by other sugars under different experimental conditions. Evidence for the nature of the pink colour was obtained from the following observations.

When mixtures of sugars and HMF were heated in 28·3 N-sulphuric acid, the spectra were often different from those of either constituent alone. Glucose (0·25 mg./ml.) at 100° showed a pale-pink colour after 3 min. heating which deepened and reached its fullest intensity before becoming brown in 6–8 min. HMF in 28·3 N-sulphuric acid became yellow-brown on heating, but a mixture of glucose and HMF became deep pink after 3 min., with a spectrum showing maxima at 470 and 515 mμ.

In a quantitative experiment, 6 ml. conc. sulphuric acid were mixed with (*a*) 1 ml. glucose + 1 ml. water, (*b*) 1 ml. HMF + 1 ml. water, (*c*) 1 ml. glucose + 1 ml. HMF. All three solutions were heated for 3 min. at 100°, when $E_{515\,m\mu}$ was found to be 0·142, 0·161 and 0·600 respectively. Thus the absorption of the mixture was almost twice that of the sum of the components. Sorbose and fructose, however, did not show any increase in pink colour with HMF. In a similar experiment, substituting fructose for glucose, $E_{510\,m\mu}$ was 0·305, 0·390 and 0·700, respectively, the last value being just that expected for a simple mixture. However, the 470 mμ. absorption was greatly increased in both ketoses on addition of HMF. Conc. sulphuric acid was mixed with fructose plus water, HMF plus water and fructose plus HMF, and allowed to stand at room temperature for 35 min., when the extinctions at 470 mμ. were 0·020, 0·096 and 0·248, respectively.

Mannose and galactose, which when heated with sulphuric acid alone (Table 2) did not even show inflexions at 515 mμ., became pink on heating for 1 min. with HMF, and then rapidly went brown (3 min.). Spectra showed sharp maxima at 515 mμ. and (more intense) at about 460 mμ.

Rhamnose plus HMF became greenish brown after 3 min. heating owing to a broad absorption band at 560–580 mμ. Arabinose and furfural showed no change.

Some sugars when mixed with HMF were found to develop the pink colour in 28·3 N-acid at room temperature. Mannose, alone among the hexoses, possessed this property. In a solution containing 0·25 mg./ml. and an equal amount of HMF, the colour was just discernible immediately after mixing with the acid, and developed further on standing. The spectrum showed maxima at about 480, 525 and 560 mμ. Lyxose rapidly became deep brown-pink ($\lambda_{max.}$ 445 and 525 mμ.) and ribose deep pink (525 mμ. only). Both spectra also showed inflexions at about 560 mμ. On heating, the colour changed rapidly and gave place to brown in 2 min. Xylose showed a pale-pink colour after standing 45 min. at room temperature.

On diluting the acid solutions threefold with water, the pink colour was destroyed. Visible spectra merely showed a gradual rise in absorption towards the ultraviolet.

Laevulinic acid in water has been shown to absorb maximally at about 265 mμ. (Singh, Dean & Cantor, 1948). In 28·3 N-sulphuric acid, a shift to 255 mμ. was observed (Fig. 2), and the spectrum did not alter on heating at 100°, apart from the transitory appearance of a weaker band at about 315 mμ.

DISCUSSION

Practical implications. Several workers have described methods for the estimation of glucose or glycogen which depend on the formation of the pink colour, measurement of which is carried out after heating for an exact time at 100°. The data presented in this paper show that the method is fundamentally unsuitable for accurate determinations because (*a*) the optical density at 515 mμ. is relatively low, (*b*) the visible spectrum consists of several absorption bands, and (*c*) the value of $E_{515\,m\mu}$ after 6 min. heating is increasing rapidly (Fig. 3), thus lowering the accuracy of the method. If heating is continued beyond 6 min., the band at 515 mμ. ceases to predominate, and the pink colour becomes masked by a deep brown which makes visual estimation impossible. Measurement of the absorption at 320 mμ. is more satisfactory as a method of estimation, since the absorption curve consists of one main maximum only, the molecular extinction coefficient is about 20 times as great, and under suitable conditions the absorption rises to a steady value (Love, 1953).

Products other than furfural or HMF. Some of the foregoing data indicate that, although sugars are converted to furfural or HMF at 60°, there is also a tendency to form other compounds with similar absorption spectra, to an extent which probably depends on the configuration of the sugar.

In the pentose series, xylose (Table 1) shows a much greater absorption at $\lambda_{max.}$ than the other sugars. When the spectra were examined, only that of xylose resembled the furfural spectrum in having a sharp maximum at 315 mμ. and a smaller sharp peak at 256 mμ. In arabinose (Fig. 1) and ribose, there was only an inflexion at 260 mμ., and in lyxose, which had by far the lowest absorption at 315 mμ., the spectrum showed an additional maximum at 290 mμ. Further, it can be seen that $\epsilon_{315\,m\mu}$ was in all cases less than that of furfural. Similarly, in the hexose series, only the spectra of glucose and fructose resembled HMF; galactose, mannose and sorbose merely showed inflexions at

260 mμ. In addition, the chief maxima of galactose and mannose were much broader than in HMF. There does not seem to be any correlation between the final optical density and the rate of reaction (Table 1), but it is evident that the only sugars producing spectra resembling furfural or HMF are those which show the greatest absorption at 315 or 320 mμ., respectively.

The conclusion to be drawn is that sugars in 28·3N-sulphuric acid may form two other compounds besides furfural or HMF, one absorbing maximally at 290 mμ., the other at about 315 mμ. The presence of the latter compound is deduced from the broadness of the main band shown in mannose and galactose and not in the pentoses. The compounds are the result of side reactions in the dehydration of the sugars and not decomposition products of furfural or HMF, since they were not observed after heating the latter substances at 60°.

Bandow (1937) showed that the furfural molecule was not radically altered in concentrated sulphuric acid, since after dilution the spectrum was identical with that of furfural in water: the shape was the same as in concentrated acid solutions, but $\lambda_{max.}$ had shifted to about 280 mμ., and the 258 mμ. band to 228 mμ. (Fig. 2). Thus the relatively high absorption at 255 mμ. obtained by the prolonged heating of glucose at 100° cannot be due entirely to HMF, since on dilution it apparently remains at about 260 mμ., although the 315 mμ. maximum moves to 280 mμ. A similar effect occurs in HMF. Identification of the substance absorbing at 255 mμ. is not possible on the strength of the available data, but laevulinic acid (Fig. 2) seems the most likely. However, the molecular extinction coefficient of the latter is too low for it to account for all the absorption.

Similarly, the high absorption at 271 mμ. produced by heating certain pentoses in 37·1N-sulphuric acid (Fig. 1) is not all due to furfural, since it does not move towards the far ultraviolet on dilution, but to 275 mμ. This band is thus probably made up of two components, furfural, which shifted from 315 mμ. on dilution, and another compound of unknown structure. The latter is not a breakdown product of furfural, since the 271 mμ. band exceeds the 315 mμ. band at all stages of heating. It will be noted that xylose again yields only furfural.

To sum up, therefore, it appears that the differences in appearance of the spectra of sugars after heating in sulphuric acid are caused by varying amounts of at least four other substances which absorb in the region 255–315 mμ. It is the formation of these substances which explains why some sugars yield less furfural or HMF than others.

The nature of the pink compound. The discovery by Blanksma & Egmond (1946) that HMF was an intermediate product in the formation of humin from hexoses might lead one to suppose that since the pink compound described by Mendel & Bauch (1926) was observable only in dilute glucose solutions, it might be formed by the condensation of just a few HMF molecules. However, this would not explain why it was formed in smaller amounts in fructose, and not formed at all in mannose, galactose or sorbose. Further, more pink colour was produced by furfural than by HMF, although neither produced much.

The result of adding pure HMF to the sugars before acidifying indicated the nature of the pink compound in a striking way. Since none of the four pentoses produced the colour alone or when mixed with furfural, but three of them did so when mixed with HMF, the $—CH_2OH$ group of HMF evidently played a part in the reaction. Also, since the pink colour developed in the cold immediately the sulphuric acid was added in lyxose, ribose and mannose, these sugars must have a structure particularly favourable for its formation. The only discernible feature common to all three sugars is that the hydroxyl groups on carbon atoms 2 and 3 are *cis* to one another. This suggests that the pink compound is formed by condensation between these groups and HMF. The fact that galactose, glucose and xylose all formed the pink colour with HMF after some delay (galactose and glucose required heating), suggested that some rearrangement of the molecules was necessary, such as epimerization, if it were possible in 28·3N-sulphuric acid.

It may be asked why among all the sugars only glucose and fructose formed the pink compound when heated alone in 28·3N-sulphuric acid. The answer is clear in the case of pentoses, for furfural, the end-product, does not condense with the hydroxyl groups. However, it was surprising that mannose, which reacted in the cold in the presence of HMF, exhibited no selective absorption at all at 515 mμ. when heated without it.

The explanation lies probably in the rate of reaction. Table 1 shows that of the three aldohexoses, glucose took the longest to be converted to HMF. If this were due to its greater stability, then, when some HMF had formed, there would still remain sufficient undecomposed glucose to react with it, whereas in the other sugars the reactive grouping would have disappeared.

Other absorption bands in the visible spectrum. The 470 mμ. band seen in the visible spectra of hexoses is probably due to the condensation of HMF with itself, since it alone is produced when HMF is heated in 28·3N-sulphuric acid (Table 2), and is the first band to form in fructose and sorbose, which are converted to HMF very rapidly (Table 1).

The remaining important band, at 430 mμ., appears last in glucose (Fig. 3) and the ketohexoses, and is the main pentose band. Since it is not formed

by furfural alone, it is probably the product of condensation of the —CHO group of furfural (HMF in hexoses) with the sugar molecule.

SUMMARY

1. The conversion of several sugars to furan derivatives at 60° in 28·3 N-sulphuric acid was followed spectroscopically. Variations in the apparent yields and in the shapes of the absorption spectra were attributed to two other absorbing compounds, which were formed at the expense of the furan derivatives in amounts dependent on the configuration of the sugars.

2. Another by-product was formed by three of the sugars when 37·1 N-sulphuric acid was used. Its absorption exceeded that of the furfural, and caused a complete change in the appearance of the spectra.

3. The colours formed when sugars were heated at 100° in 28·3 N-sulphuric acid were found to be due to three main absorption bands. The nature of the absorbing substances is discussed.

The work described in this paper was carried out as part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research.

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Studies on Influenza Virus Receptor-Substance and Receptor-Substance Analogues

I. PREPARATION AND PROPERTIES OF A HOMOGENEOUS MUCOID FROM THE SALIVARY GLAND OF SHEEP

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Agglutination of red cells by influenza virus, first described by Hirst (1942), may be inhibited by specific antibody, by non-specific serum inhibitors (Burnet & McCrea, 1946; McCrea, 1946) and by a wide range of mucoid substances. Relatively weak inhibition of viral haemagglutination by apple pectin was described by Green & Woolley in 1947, but this apparently non-specific effect has not been confirmed by other workers. Inhibition by serum mucoid and crude human 'mucins' (Burnet, McCrea & Anderson, 1947) appears, however, to be a more specific reaction, since the inhibitory power of such substances is destroyed on incubation with living influenza virus or the bacterial receptor-destroying enzymes (Burnet, McCrea & Stone, 1946; Burnet & Stone, 1947) in the same highly characteristic fashion as are the 'true' receptors on the erythrocyte surface.

Although purified human serum mucoid (McCrea, 1948*a*; Hirst, 1949) and ovarian-cyst mucopolysaccharide (McCrea, 1948*b*) show high activity in inhibiting haemagglutination, it has not yet been definitely established that such activity is an integral part of the mucoid molecule. Other mucoid substances such as ovomucin (Lanni & Beard, 1948; Gottschalk & Lind, 1949), human cervical mucin (Burnet, 1948*a*) and the allantoic-fluid inhibitor (Svedmyr, 1948), which show high antihaemagglutinin activity, have generally been used in a relatively crude form. Until a virus-inhibitory mucoid has been obtained in a homogeneous state,

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therefore, it might well be argued that the anti-haemagglutinin activity of such substances is due to some contaminating factor.

Anderson (1950) observed that a thermostable inhibitor of influenza virus haemagglutinin was present in the salivary glands of ferrets and sheep, and an extremely active electrophoretically homogeneous mucoprotein can readily be isolated from the latter source (McCrea, 1951). This mucoprotein has now been studied in greater detail and the present paper describes the preparation of a substance which moves as a single, sharply defined component on both electrophoresis and ultracentrifugation. Further, the mucoid shows exceptionally high anti-haemagglutinin activity against influenza B virus and moderate activity against certain influenza A strains. An essentially homogeneous mucoprotein recently isolated from urine (Perlmann, Tamm & Horsfall, 1952) shows activity of closely similar order.

METHODS

Analytical methods

Nitrogen was determined by the micro-Kjeldahl method in a Markham still (Markham, 1942), phosphorus by the method of Briggs (1922), reducing sugar by the method of Somogyi (1937), and methyl pentose (fucose) according to the method of Dische & Shettles (1948) in a Uvispek spectrophotometer. *N*-Acetylhexosamine was determined by a modification of the colorimetric method of Morgan & Elson (1934), hexosamine by a modification of the method of Elson & Morgan (1933) and also by the method of Dische & Borenfreund (1950). In spite of careful purification of all the reagents used in the latter procedure, including repeated crystallization and sublimation of the indole, high absorption occurred in the blank solutions; in our hands this method was less sensitive and more cumbersome than the Elson & Morgan (1933) procedure. Derivatives of the hexosamine component obtained on acid hydrolysis were prepared by the methods of Jolles & Morgan (1940) and Annison, James & Morgan (1951).

Paper chromatography was carried out for amino acids as described by Consden, Gordon & Martin (1944) and for sugars and amino sugars as described by Partridge (1948). Whatman no. 4 paper was used throughout.

Titration of viral antihaemagglutinin

The antihaemagglutinin activity of the preparations was determined as described by Anderson (1948). A series of double dilutions of mucoid was made in 0·2 ml. of physiological saline, an equal volume of saline containing 5 agglutinating units of influenza virus was added to each tube, and, after standing at 4° for 1 hr., a further volume of 1% (v/v) sensitive fowl red cells (Anderson, 1948) or pigeon cells was added. The inhibition titre was read from the pattern of deposited cells after standing for 1 hr. at 4°. The 'indicator' forms of influenza A and B virus were prepared by heating allantoic fluid under the conditions described by Stone (1949*a*). The virus strains, which were kindly provided by Dr A. Isaacs, of the World Influenza Centre, Mill Hill, London, were reference strains cultivated by the standard suballantoic method. Purified receptor-destroying enzyme of *Vibrio cholerae* (Burnet & Stone, 1947; Ada & French, 1950) was obtained through the courtesy of Sir MacFarlane Burnet.

EXPERIMENTAL

Isolation of the purified mucoid

Extraction of salivary glands. The material used in the present series of experiments was obtained from sheep which had been inoculated 4 days previously with vaccinia virus. Immediately after the sheep had been killed, the submandibular glands were excised and dehydrated for 2–3 months in several changes of absolute ethanol. The glands were then minced finely, air-dried for several hours at room temperature, and extracted with water in a Waring Blendor, allowing approximately 300 ml. of water to each 100 g. glands. The suspension was brought to 80° for 2 min., cooled to room temperature, and shaken for 6 hr. After standing overnight at 4° in the presence of $CHCl_3$, the insoluble debris was removed by filtration and finally by centrifugation at 8000 rev./min. in a Servall angle centrifuge (model XL), and the supernatant fluid dried from the frozen state. Only a small amount of active material could be obtained on further extraction of the gland debris. The average yield of crude mucoid was 30 g./kg. of dry glands.

In earlier experiments (McCrea, 1948*b*), highly active virus-inhibitory mucoid was prepared by extracting freshly excised submandibular glands of normal sheep without preliminary dehydration in ethanol. Such material was closely similar in activity and chemical composition to that described in the present paper, but was markedly polydisperse in the ultracentrifuge. Some preparations were made by precipitation at 4° with acetic acid; a firm clot closely resembling hyaluronic acid was formed, but this procedure yielded material of low activity and destroyed physical properties such as the viscosity and 'spinnbarkeit' characteristic of crude salivary mucoid obtained by other methods. Significant loss of activity also occurred on treatment with trichloroacetic acid in the cold.

Fractionation with ethanol. In a typical experiment, a 10% (w/v) aqueous solution of crude mucoid was cooled to 4° and ethanol run in slowly with constant stirring in the presence of potassium acetate. The fraction separating between 40 and 66% (v/v) ethanol concentration, which contained practically all the activity, was collected on the centrifuge, dialysed against distilled water at 4°, and dried from the frozen state; yield 47% of the original weight (fraction *A*). A second fraction (fraction *B*) collected between 75 and 80% (v/v) ethanol concentration represented 17% of the original material. Fraction *A* dissolved slowly to form a turbid viscous solution; fraction *B* dissolved almost instantaneously giving a water-clear solution.

Treatment with chloroform-butanol mixture. In one experiment, 3·4 g. of fraction *A* were dissolved in 320 ml. of Palitzsch's borate buffer, pH 6·8, 140 ml. of 9:1 (v/v) chloroform-butanol mixture was added, and the emulsion stirred vigorously for 1 hr. The resulting gel was separated by centrifugation, and the procedure repeated until no

further insoluble material formed. After dialysis, the supernatant fluid was dried from the frozen state, yielding 1·3 g. of snow-white powder (fraction *C*I). No decrease in activity occurred.

In another experiment 13·5 g. of fraction *A* were treated similarly with the exception that borate buffer was replaced by 0·2M-acetate buffer, pH 4·7. As will be described in a later section, the most completely homogeneous material (fraction *C*II) was obtained in this way.

Properties of the purified mucoid (fractions C*I and* C*II)*

Physical properties. Fractions *C*I and *C*II dissolved rapidly in water giving water-clear non-viscous solutions. The mean specific rotation was $[\alpha]_{5461} + 18°$ in water at room temperature. The ultraviolet-absorption spectrum examined in a Uvispek spectrophotometer showed a well defined absorption peak at 255–260 mμ. (Fig. 1), presumably due to the presence of nucleoprotein.

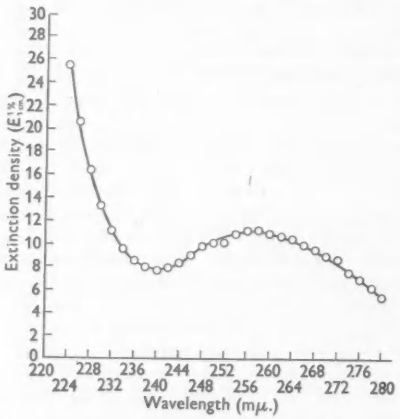

Fig. 1. Ultraviolet-absorption spectrum of fraction *C*I; 0·05 % (w/v) solution in 0·85 % (w/v) NaCl.

The physical homogeneity of the preparations was examined in the ultracentrifuge and electrophoresis apparatus; details of these studies are given in the addendum by Mr E. A. Caspary. In summary, all preparations migrated as one sharply defined component on electrophoresis, fraction *C*II remained as a single component even after prolonged centrifugation at full speed (270 000 *g*) in the ultracentrifuge, whilst fraction *C*I showed two closely related peaks after prolonged centrifugation.

Chemical properties. No precipitate occurred when equal volumes of the following reagents were added to a 2 % (w/v) aqueous solution of fractions *C*I or *C*II: trichloroacetic acid (20 %), sulphosalicylic acid (10 %), phosphotungstic acid (2 %), tannic acid (5 %), picric acid (5 %), basic lead acetate (2 %) (all w/v). There was no precipitation on boiling. The ninhydrin and biuret tests were strongly positive, the Millon test weakly positive; some preparations gave a weak Molisch reaction. The Dische (1947) test for hexuronic acid was negative.

Analytical figures for a typical preparation (fraction *C*I) were: N, 10·1 %; P, 0·4 %; S, 1·4 %*; ash 1·4 %. The phosphorus content varied considerably in various preparations (one sample contained no detectable phosphorus), but the other figures did not change significantly. The maximum amount of reducing sugar, calculated as glucose and

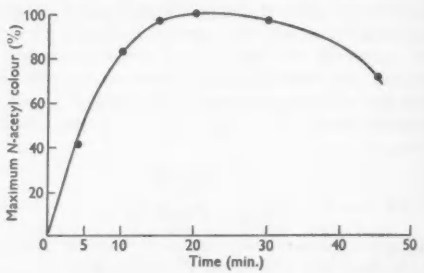

Fig. 2. Development of *N*-acetylhexosamine colour reaction on heating purified mucoid *C*I with 0·05 N-Na_2CO_3 at 100°.

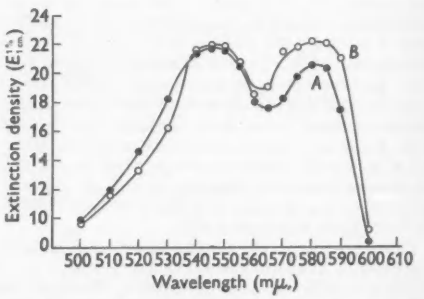

Fig. 3. Absorption spectra of the colour produced by purified mucoid, fraction *C*I (*A*), and pure *N*-acetylglucosamine (*B*), after heating with 0·05 N-Na_2CO_3 and treatment with Erhlich's reagent.

released after 48 hr. hydrolysis in 0·5 N-hydrochloric acid, was 13·6 %. The mucoid gives the colour reaction for *N*-acetylhexosamine on heating with 0·05 N-sodium carbonate and treatment with Ehrlich's reagent (Morgan & Elson, 1934), the maximum colour obtained being equivalent to 4·1 %, after heating at 100° for 20 min. (Fig. 2). The absorption spectrum of the colour obtained in the *N*-acetylhexosamine test, which shows two well

* S determined by Weiler and Strauss, Oxford.

defined maxima, corresponded closely to that of a known specimen of *N*-acetylglucosamine (Fig. 3) (Aminoff, Morgan & Watkins, 1952).

After hydrolysis in 5 N- or 0·5 N-hydrochloric acid, the maximum hexosamine, calculated as glucosamine base, was 12·4 % (Elson & Morgan, 1933), or 16·7 % by the method of Dische & Borenfreund (1950). About 1 % methyl pentose (fucose), determined by the method of Dische & Shettles (1948), was present in all preparations.

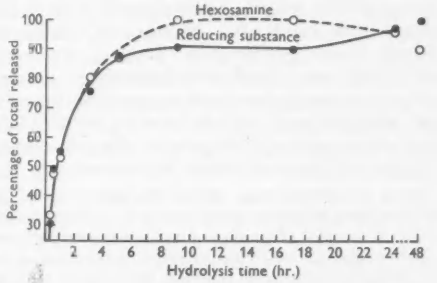

Fig. 4. Release of reducing substances (calculated as glucose) and hexosamine (as glucosamine base) on hydrolysis of fraction *C*I with 0·5 N-HCl at 100°.

Hydrolysis curve. Release of reducing substances and hexosamine was followed by sealing samples of a 1 % (w/v) aqueous solution of fraction *C*I with an equal volume of N-hydrochloric acid in small ampoules, and immersing in a water bath at 100°. The ampoules were removed at the intervals shown on the curve (Fig. 4), the contents neutralized, and determinations carried out in triplicate by the methods described. The curve shows that the maximum amount of hexosamine (12·4 %) was released after hydrolysis for 9 hr., and decreased slightly after 17 hr.; at 9 hr. 90 % of the total reducing substances have been released, but the maximum (13·6 %) was not reached until 48 hr.

Isolation of hexosamine derivatives

Attempts were made to isolate crystalline derivatives of the hexosamine component released on hydrolysis of purified mucoid with acid. With this mucoid, however, it appears that it is much more difficult to obtain such compounds than had been found with blood-group A substance (Annison *et al.* 1951).

In one experiment, fraction *C*II (500 mg.) was hydrolysed at 100° for 16 hr. with 0·5 N-HCl under a stream of N_2. The hydrolysate was taken to dryness *in vacuo* and dissolved in 10 ml. of 0·4 M-borate buffer at pH 9, according to the procedure of Annison *et al.* (1951). The solution was cooled to 0°, the pH adjusted to 9·5 by the addition of NaOH, and 3 ml. of 1:2:4-fluorodinitrobenzene added in ethanol solution. After standing for 3 days at 0°, the dark-orange solution was evaporated to dryness *in vacuo* at room temperature. A few drops of amyl alcohol:chloroform mixture were added and the moistened powder transferred to the top of a 50 g. kieselguhr column, and developed in 30 % (v/v) amyl alcohol:chloroform mixture at 2°. After discarding some fast-moving impurity, a slow-moving orange band was collected. This was concentrated to dryness *in vacuo* at 40° and the material was run twice more on kieselguhr columns in the same way. The product, taken to dryness *in vacuo*, was crystallized twice from 80 % (v/v) acetone-water mixture, and once from 0·5 ml. of acetone at −10°. A small yield (3 mg.) of orange crystals was obtained, m.p. (uncorrected) 160–162°. An authentic sample of *N*-2:4-dinitrophenyl galactosamine melted at 170–172°. The experiment was repeated twice with similar results.

An attempt was also made to obtain a 2-hydroxynaphthaldehyde derivative of the hexosamine (Jolles & Morgan, 1940). Fraction *C*I (400 mg.) was hydrolysed as before, taken to dryness *in vacuo* over NaOH, and treated with excess (400 mg.) 2-hydroxynaphthaldehyde. The crude Schiff's base was taken up in a minimum of warm methanol, treated with acetone, and stood for several days at −10°. No crystals separated, however, even after 'seeding' with 2-hydroxynaphthylidenegalactosamine. Satisfactory yields of the crystalline derivatives of glucosamine and galactosamine were obtained when the pure hexosamines were treated in the same way.

Chromatographic examination of the hydrolysed mucoid

Portions of the hydrolysed mucoid were examined for sugars, hexosamines, and amino acids by the technique of paper chromatography.

Hexosamine. One cherry-red spot was obtained when the hydrolysed material was run in collidine and sprayed with the hexosamine reagents (Partridge, 1948); the spot showed the same R_F value (0·28) as 'known' galactosamine run on the same paper strip. A further spot appeared ahead of the 'unknown' spot if glucosamine was added to the hydrolysate, but not if galactosamine was added (cf. Aminoff & Morgan, 1948). This effect was best shown if the chromatograms were run for 48 hr. at 4°.

Sugars. Hydrolysed material (0·5 N or 5 N-HCl) was run in collidine, butanol:acetic acid and phenol:ammonia, and sprayed with the silver nitrate (Partridge, 1948) and aniline hydrogen phthalate (Partridge, 1949) reagents. Apart from the hexosamine spot, however, no clearly defined spots were obtained.

Amino acids. Material hydrolysed with 6 N-HCl was examined by two-dimensional chromatography, the solvents being collidine and phenol (Consden *et al.* 1944). The following amino acids, and chondrosamine, were identified: aspartic acid, glutamic acid, glycine, arginine, serine, alanine, threonine, valine, leucine and isoleucine.

Virus antihaemagglutinin activity

Inhibition spectrum. A 1 % (w/v) aqueous solution of fraction *C*I was titrated against a number of active and indicator influenza virus strains using chicken red cells. The results are shown in Table 1.

It will be seen that this preparation shows extremely well the Francis phenomenon (Francis, 1947), namely that indicator influenza B virus, strain LEE, heated to 56° for 30 min., is very much more sensitive to inhibition than is the active unheated form: in the present instance there is an almost 1000-fold increase in titre against the indicator virus. With the influenza A virus strains, PR 8 shows an approximately tenfold increase in sensitivity to inhibition after conversion to the indicator form, whilst strain MEL, as was previously observed by Stone (1949*b*) is inhibited to only a slight extent by sheep salivary mucoid. Swine influenza (Shope strain 15) is also inhibited to relatively low titre. Fraction *B* (precipitated between 75 and 80 % ethanol concentration) inhibited heated LEE to a titre of 20 000. Fraction *C*II was not examined against all virus strains, but showed activity of the same order as fraction *C*I against LEE, H. LEE, PR 8, and H.PR 8.

Table 1. *Antihaemagglutinin activity of purified mucoid, fraction C*I*, in* 1 % *(w/v) aqueous solution*

Virus strain	Titre*	Virus strain	Titre*
LEE, active	4 800	MEL, active	10
LEE, indicator	3 200 000	MEL, indicator	150
PR 8, active	400	SW, active	180
PR 8, indicator	5 600	SW, indicator	800

* Reciprocal of dilution giving 50 % neutralization of virus.

Effect of various treatments

Treatment with liquid phenol. Crude mucoid (300 mg.) was taken up in 13 ml. of 90 % (w/v) phenol (Morgan & King, 1943). The bulk of the material dissolved rapidly, and after stirring for 6 hr. only 6·2 mg. of insoluble material separated on centrifugation. A 1:1 (v/v) mixture of ethanol and phenol was cautiously added to the supernatant fluid; no precipitate formed at 50 % but a heavy precipitate separated between 55 and 75 % (v/v) ethanol concentration. The precipitate was collected by centrifugation, washed free from phenol with ethanol, taken up in water, and freeze-dried; 25·9 mg. of grey-white material was obtained. The material remaining in solution at 75 % ethanol concentration was dialysed free from phenol and freeze-dried.

The activity of the fraction precipitated with 75 % ethanol was reduced by approximately 80 % as compared with the starting material which, in 1 % (w/v) aqueous solution, inhibited H. LEE to a titre of $1{\cdot}2 \times 10^6$. The activity of the phenol-insoluble material and the fraction soluble in 75 % ethanol was reduced by approximately 95 %. Inactivation to a similar extent occurred if crude mucoid was dissolved in 90 % phenol, stirred for several hours, dialysed free from phenol and dried from the frozen state.

Action of trypsin. The virus-inhibitory activity of the purified mucoid is destroyed extremely rapidly on incubation with dilute trypsin.

In one experiment purified mucoid was incubated at 37° with crystalline trypsin (final concentration 1 μg./ml.) in borate buffer pH 7·5. Samples were removed at intervals, heated to 100° to inactivate the enzyme, and titrated in the usual manner against indicator LEE virus. Control tubes without enzyme and with heat-inactivated enzyme were included.

After incubation for 15 min. the titre of the enzyme-treated material was reduced to 17 % of the control titre, and after 30 min. no inhibitory activity could be detected. Loss of inhibitory activity was most rapid at the beginning of incubation, since a sample removed at zero time (immediately after adding the enzyme solution) showed only 33 % of the activity of the control. Experiments of this type will be described in detail elsewhere.

Action of metaperiodate. Equal volumes of potassium metaperiodate diluted in series from 0·01 to 0·001 M were added to samples of purified mucoid dissolved in 0·2 M-acetate buffer, pH 5·5 (Walpole, 1914). After standing for 1 hr. at 20° the contents of the tubes were titrated in 0·1 M-glycerol saline against indicator LEE virus.

Even with the highest dilution of metaperiodate (final concentration 0·00033 M), the titre of the mucoid was reduced to 2 % of the control value.

Action of receptor-destroying enzymes. Inactivation of partially purified sheep salivary mucoid by the receptor-destroying enzyme (RDE) of *V. cholerae* and active influenza virus was studied in detail by Stone (1949*b*). The mucoid obtained in the present investigation is also rapidly inactivated by RDE, as may be illustrated by an experiment in which highly purified *V. cholerae* RDE (0·001 % w/v in Walpole's 0·2 M-acetate buffer, pH 6·2) was incubated at 37° with a solution of fraction *C*I. The titre (H. LEE) was reduced by 99 % after incubation for 1 hr.

DISCUSSION

The homogeneity of the isolated mucoid (fraction *C*II) is well established: a single sharply defined component is revealed both on electrophoresis and after prolonged ultracentrifugation. There seems little doubt, therefore, that in this instance the virus-inhibitory factor is a component of the mucoid molecule, although it does not necessarily follow that more than a small proportion of the molecule is concerned in inhibiting haemagglutination. With purified ovomucin, for example, it has been shown that antihaemagglutinin activity is concentrated in as little as 5–10 % of the total molecule (Fazekas de St Groth & Gottschalk, 1951).

The inhibitory activity of the purified mucoid against indicator influenza B virus is at least as high as that of any mucoid virus inhibitor previously

recorded. A titre of the order of 3×10^6 obtained by serial dilution under the conditions described means that in the 'end-point' tube, in which 50 % of the virus has been neutralized, the final concentration of mucoid is of the order of 0·001 μg./ml. This amount of mucoid is apparently capable of combining with several thousand virus particles, thus preventing their adsorption to the erythrocyte surface with consequent haemagglutination. Antihaemagglutinin titre against active (unheated) LEE virus is approximately one-thousandth of that against the indicator form, but even so is higher than was observed for 'specific' receptor-substance mucoproteins extracted from human erythrocytes (McCrea, 1952, 1953). The inhibitory activity of a purified mucoprotein from human urine is closely similar to that of the submandibular mucoid, but the former differs in several physical respects such as high viscosity, slow solubility in water, insolubility in 0·05 M-sodium chloride, and in having a high molecular weight of the order of $7{\cdot}0 \times 10^6$ (Tamm & Horsfall, 1950, 1952).

The nature of the hexosamine component of the salivary mucoid is not definitely established; it is certainly not glucosamine, but yet does not entirely correspond to galactosamine. The R_F value of the unknown hexosamine is identical with that of galactosamine as determined by paper chromatography, but this alone is not sufficient evidence for its identification. The fact that the *N*-acetylhexosamine content, as judged from the Morgan & Elson (1934) reaction, is only approximately one-third of that expected from the hexosamine colour obtained after acid hydrolysis, suggests the presence of galactosamine rather than glucosamine because the intensity of the *N*-acetylhexosamine colour of the former substance is considerably less than its hexosamine colour (Aminoff *et al.* 1952). The melting point of the crystalline *N*-2:4-dinitrophenyl hexosamine obtained was 10° and 20° lower than for authentic *N*-2:4-dinitrophenyl galactosamine and *N*-2:4-dinitrophenyl glucosamine respectively. Whilst this may be due to the fact that on hydrolysis with 0·5 N-acid the hexosamine was not liberated entirely free from other degradation products, it is possible that a new hexosamine is present. The finding that the Schiff's-base derivative was considerably more soluble than the authentic glucosamine or galactosamine derivatives is perhaps evidence in the same direction. In a note published during the course of the present work, Blix, Svennerholm & Werner (1950) state that human saliva and serum mucoid contain galactosamine only. Other receptor analogues such as ovomucin and ovarian cyst polysaccharide appear to contain both glucosamine and galactosamine, as do also inhibitory mucoproteins isolated from human erythrocytes (McCrea, 1952, 1953).

Finally, the relation of the soluble mucoid 'receptor analogue' to the red-cell receptor should briefly be considered. In its sensitivity to trypsin and metaperiodate, and almost complete solubility in and inactivation by liquid phenol, sheep submandibular mucoprotein resembles the serum mucoid antihaemagglutinin (McCrea, 1948*a*, Hirst, 1949) and ovomucin (Gottschalk & Lind, 1949) rather than ovarian cyst mucoid which is phenol-insoluble and relatively resistant to the action of proteolytic enzymes. Virus-inhibitory mucoproteins isolated by various methods from human erythrocytes are phenol-soluble but resistant to trypsin (McCrea, 1952, 1953). The action of metaperiodate and proteolytic enzymes on the homogeneous virus inhibitor of human urine (Tamm & Horsfall, 1952) has not yet been described. Sheep salivary mucoid is progressively inactivated by living influenza virus (Stone, 1949*b*), by receptor-destroying enzymes, and by the periodate ion, properties which are all duplicated by the virus receptor on the intact red-cell surface. Inactivation by living virus of both the cell receptor and soluble mucoids is a specific enzymic reaction (Hirst, 1948; Burnet, 1948*b*; Stone, 1949*b*; Tamm & Horsfall, 1952), which takes place under similar conditions and with the same end result, namely loss of the capacity to adsorb further virus. It may, therefore, be concluded that similar chemical groupings exist in the erythrocyte receptor and the mucoids, and that the virus-inhibition phenomenon is in essence a form of substrate competition. The exceptionally high activity of sheep salivary mucoid may thus be due to a greater proportion, or availability, of those groupings which are enzymically modified by living virus and to which indicator virus remains firmly adsorbed. The precise nature of the chemical groupings involved is not yet known; apart from the amino acids found in all these mucoids, the only common factor appears to be hexosamine. An alkali-labile *N*-acetylhexosamine-amino-acid complex is released as the split product after incubation of ovomucin with influenza virus (Gottschalk, 1949, 1951), a finding which suggests that the hexosamine component of this receptor analogue may be involved in the enzymic action of influenza virus.

SUMMARY

1. The isolation from the submandibular glands of sheep of a mucoprotein with high antihaemagglutinin activity against influenza B virus is described.

2. The mucoprotein was homogeneous on electrophoretic and ultracentrifugal examination. Analysis gave the following figures: N, 10·1; P, 0·4; S, 1·4 and fucose 1·1 %. On acid hydrolysis 13·6 % of reducing substances and 12·4 % of hexosamine were released. The ultraviolet-absorption spectrum indicated the presence of nucleic acid.

3. One hexosamine was revealed by paper chromatography; the R_F value was identical with that of galactosamine, but the nature of the hexosamine has not definitely been established. The hydrolysate contained ten amino acids.

4. Agglutination of red cells by indicator influenza B (LEE) virus was inhibited by the purified mucoid at a concentration of 0·001 μg./ml.

5. Antihaemagglutinin activity was lost on treating the mucoid with receptor-destroying enzymes, potassium periodate and trypsin.

6. The relation of the mucoid to red-cell virus receptors and other soluble mucoid antihaemagglutinins is discussed.

I am greatly indebted to Prof. W. T. J. Morgan, F.R.S., for advice, to Dr E. F. Annison for assistance with the isolation of dinitrophenyl hexosamine, and to Dr D. McClean for collecting the salivary glands. I wish also to express my thanks to Sir Alan Drury, F.R.S., for hospitality at the Lister Institute of Preventive Medicine, and to the Australian National University for a scholarship.

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ADDENDUM

Physicochemical Examination of a Mucoid from the Salivary Gland of Sheep

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The sheep salivary mucoid was examined in the electrophoresis apparatus and ultracentrifuge, and in addition the diffusion constant and partial specific volume were determined.

METHODS

Electrophoresis. The material was examined in a Tiselius electrophoresis apparatus at 0° using the diagonal-schlieren optical system and monochromatic light $\lambda = 546$ mμ., isolated from the mercury arc by a suitable filter. Solutions were made up in suitable buffers to give concentrations of 1·5–2·0% (w/v) and dialysed for 24 hr. against buffer. These concentrations were chosen in order to detect traces of contaminating high-molecular-weight substances.

Sedimentation. Ultracentrifugal examination was carried out in a Svedberg oil turbine machine at 54 000 rev./min. (240 000 *g*) using a diagonal-schlieren optical system (Philpot, 1938). The variation of sedimentation constant with concentration was examined over the range 0·5–1·0% (w/v). Solutions were made up in suitable buffers with added NaCl to suppress charge effects on sedimentation.

Diffusion. The diffusion measurements were carried out at 25° by the Gouy interferometric method (Kegeles & Gosting, 1947) in a modification of the apparatus of Gosting, Hanson, Kegeles & Morris (1949). In order to increase the sensitivity of the method the blue line, $\lambda = 436$ mμ. isolated by a suitable filter from a high pressure mercury arc, was used.

Partial specific volume. This was determined pyknometrically at 25°, the mucoid being dissolved in the buffer used for the sedimentation measurements.

RESULTS

In both phosphate (pH 8, $I = 0{\cdot}2$) and acetate (pH 4, $I = 0{\cdot}1$) buffers only one component could be demonstrated after prolonged electrophoresis. At pH 8 the ascending boundary was symmetrical, the descending side showed some asymmetry. At pH 4 both boundaries were slightly skewed (Fig. 1). The spreading of both boundaries appeared similar at both pH's 4 and 8. Migration was anodic in both cases, though somewhat slower at pH 4.

In the ultracentrifuge at pH 8 only one component was demonstrable, the peak remaining relatively sharp even after 150 min. at 240 000 *g* in a 0·75% (w/v) solution, and at 0·5% (w/v) a satisfactory peak was present after 90 min. at 240 000 *g* (Fig. 1). Sedimentation constants determined at three concentrations showed a slight, apparently linear, variation of sedimentation constant with concentration (Table 1).

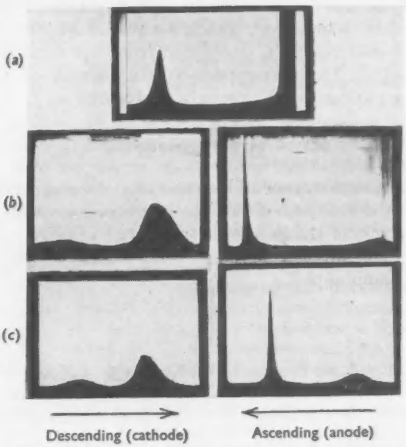

Fig. 1. Sedimentation diagram and electrophoresis patterns of sheep salivary mucoid. (*a*) Sedimentation after 105 min. at 240 000 *g*, 0·75% (w/v) in phosphate-NaCl (phosphate, pH 8, $I = 0{\cdot}2$; NaCl, 0·15M). (*b*) Electrophoresis pattern after 37·5 mA-hr. of 1·2% (w/v) solution in acetate, pH 4, $I = 0{\cdot}1$. (*c*) Electrophoresis pattern after 27·5 mA-hr. of 1·0% (w/v) solution in phosphate, pH 8, $I = 0{\cdot}2$. Lower arrows show direction of migration in both (*b*) and (*c*).

Table 1. *Sedimentation and diffusion constants of sheep salivary gland mucoid*

(Values of sedimentation and diffusion constants corrected to water at 20° (Svedberg & Pedersen, 1940).)

Concentration (g./100 ml.)	$S_{20} \times 10^{13}$ (corr.)	$D_{20} \times 10^{7}$ (corr.)
1·00	1·99	—
0·75	2·28	—
0·50	2·53	2·27
0·25	—	2·31

The diffusion constant was measured at pH 8 and concentrations 0·5% (w/v) and 0·25% (w/v). The values of C_t (the maximum downward displacement of light at the photographic plate if the light followed

geometrical optics, cf. Longsworth, 1947; Gosting & Morris, 1949) show a pronounced tendency to decrease as the fringe number j increases, indicating a departure from the ideal form, defined by the probability integral. Calculations were made from the fringe $j=1$, this would approximate to D_A (diffusion constant determined by height-area method).

The partial specific volume of the substance was 0·691.

The molecular weight was calculated from the usual equation (Svedberg & Pedersen, 1940), using the sedimentation and diffusion constant values obtained in a solution containing 0·5 g./100 ml. mucoid and the value for the partial specific volume, already determined, giving a value of 87 000. The frictional ratio f/f_0 calculated from the same data was 1·51. This corresponds to an axial ratio of 10 or may indicate some degree of hydration.

DISCUSSION

The results appear to indicate that electrophoretically the mucoid is essentially homogeneous, and, the nitrogen of the mucoid is therefore an integral part of the molecule, and not attributable to protein contaminants.

The appearance of the ultracentrifugal boundary indicates a fairly homogeneous material, taking size and shape into consideration. Some deviation from an ideal relationship is shown in the diffusion measurements, which is not large, but suggests the presence of more than one molecular species. The molecular size and degree of asymmetry are considerably smaller than those reported by Tamm & Horsfall (1952) for a urinary mucoid of similar biological properties.

The conclusion is that the mucoid is slightly polydisperse, but the precise range and distribution of molecular size have not been calculated, since insufficient data were available.

SUMMARY

1. The mucoid has been shown to be essentially homogeneous electrophoretically.

2. The molecular weight of the mucoid calculated from sedimentation and diffusion data is 87 000 with a frictional ratio of 1·51.

3. The mucoid is probably slightly polydisperse and not greatly asymmetrical in molecular shape.

I wish to acknowledge the technical assistance of Mr H. Murray.

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The Proportion of Phosphatase Activity Demonstrable in Brain by Histological Techniques

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Characteristic differences have been found in brain tissue in the histological sites of hydrolysis of adenosine triphosphate (ATP), adenosine 5-monophosphate (A5MP), aneurin pyrophosphate (APP), and of glycerophosphate (GP) by the acid and alkaline phosphatases (Naidoo & Pratt, 1951, 1952). Evidence that separate enzymes were represented was provided by differences in the conditions needed and by the preferential suppression of each reaction by suitable inhibitors. The validity of these results would be more readily assessable if the proportion of the potential enzyme activity of the tissue they represented was known. This is the purpose of the present work.

Histological study of the sites of phosphatase activity is made possible by the precipitation of inorganic phosphate at the site of its formation. The hydrolysis of a suitable substrate in the presence of

either lead or calcium ions, according to the pH of the medium, produces a precipitate which is converted to a coloured compound in order to make possible a microscopical examination. Earlier workers have applied these methods most frequently to tissue fixed by treatment with acetone or ethanol. Danielli (1946, 1950) has drawn attention to the danger of incorrect localizations in such procedures, due to diffusion of enzymes or of reaction products and to the possibility of enzyme loss which may not be uniform; other workers have confirmed that such losses may be considerable (Doyle, 1948; Rabinovitch, Junquiera & Fajer, 1949). The acid and alkaline phosphatase activities remaining in tissues at various stages of acetone or ethanol fixation and paraffin-wax embedding were determined by Stafford & Atkinson (1948) who found that the alkaline phosphatase activity of prepared sections had fallen to 20–30 % and that of acid phosphatase to about 5 % of the original value. Comparable values were obtained by Berenbom, Yokoyama & Stowell (1952) in liver after procedures involving acetone treatment.

A large proportion of the activity of five brain phosphatases was represented in histological procedures using freeze-drying instead of chemical fixation (Pratt, 1952). Assessment of activity by direct examination of the stained sections was rejected because of the irregular distribution of the deposit. Instead, the inorganic phosphate precipitated in the sections was estimated directly, making use of the Martin & Doty (1949) phosphomolybdic acid extraction by an organic solvent; the inorganic phosphate precipitated in a single microtome section after incubation in a histological medium could be estimated thus. Standards for comparison were provided by estimation of the activity of saline suspensions of fresh tissue by ordinary biochemical methods. Doyle (1950) has described a method for the estimation of lead sulphide deposits in sections after alkaline phosphatase staining. This method should be applicable after other phosphatase procedures, but direct estimation of the phosphate was preferred and was convenient also for the estimation of fresh tissue activity.

Four separate levels of enzyme activity must be considered: (1) the activity of a fresh tissue suspension under optimal conditions; (2) the activity of prepared tissue sections in the present histological procedure; (3) the activity of a fresh tissue suspension under the conditions of the histological method; (4) the activity of tissue subjected to chemical fixatives during its histological preparation, including paraffin removal. Work to which reference was made above (Doyle, 1948; Stafford & Atkinson, 1948; Rabinovitch *et al.* 1949; Berenbom *et al.* 1952) has shown that level (4) was considerably below the activity of fresh tissue. In the present work comparisons have been made between levels (2) and (3) in order to detect enzyme loss in the histological preparation of the tissue or in the incubation procedure which might reduce the proportion of the enzyme demonstrated. This was believed to be more important than comparison with the level (1) which involved in addition such factors as the effects of the cations which were necessarily present to precipitate inorganic phosphate.

EXPERIMENTAL

Wistar rats, 40–100 days old, were used. The histological material was prepared as described by Naidoo & Pratt (1951). The principal steps were the removal of the brain under ether anaesthesia, rapid freezing of 2 mm. coronal slices at $-40°$, and drying of the tissue under vacuum below $-30°$. The dried material was embedded in melted paraffin wax at 52–56° for 20 min., the pressure being allowed to rise sufficiently to avoid boiling of the wax. Serial sections were cut 15 μ. thick, mounted dry on albuminized glass slides and de-waxed by treatment with three changes of light petroleum of which the last, *iso*pentane, was allowed to evaporate before immersing sections in the incubation media.

Sections were weighed on a quartz-fibre balance before the removal of the wax in order to check their thickness and uniformity. The area of the tissue was measured on the cut surface of the block. Tracings were made on a Vickers projection microscope and the areas measured by a planimeter. Sections were cut by a Reichert rotary microtome. In order to obtain uniformity, it was essential to cut serial sections, turning the handle at a slow steady rate without a break, rejecting the first section, and any which appeared to be abnormal. The mean thickness in a series of thirty consecutive sections was 14·8 μ. (S.D. of one section, $\pm 0{\cdot}40\,\mu$.). The weight of fresh tissue was usually 0·5–1·5 mg./section. It was necessary to use three medulla sections to provide sufficient tissue for one determination.

Glass-distilled water was used throughout. The solutions of substrates were prepared as in earlier work, except that a more concentrated (0·005 M) ATP stock solution proved more convenient and acetone precipitation of the APP was advantageous in order to reduce the inorganic PO_4 content of the solution. The silicotungstic acid was freed from inorganic PO_4 by ether extraction (cf. North, 1939). Suitable blanks were included for each procedure.

Enzyme activity in sections. Slides (three to five), each bearing a pair of adjacent sections, were incubated at 37·5° with gentle agitation in 30 ml. of medium contained in a Coplin staining jar. One slide was removed after a minute (the others at various intervals) and rinsed in water (10 sec.). Repetition of this rinse or the use of dilute lead acetate solution in place of water yielded similar inorganic PO_4 values. The sections were allowed to drain but not to dry. One section from each pair was reserved for visual examination. The other was scraped from the slide by means of a safety razor blade held at an angle of about 60° to the glass. The section, rolled upon the cutting edge of the blade, was transferred by means of a finely drawn glass rod to a glass-stoppered test-tube containing extraction fluid, and also the

Table 1. *Composition of media for the incubation of sections*

Substrate	Concn. (M)	pH	Buffer	Buffer concn. (M)	Lead acetate (M)	$CaCl_2$ (M)	$MgCl_2$ (M)
GP	0·0100	5·3	Acetate	0·050	0·002	—	0·0033
GP	0·0200	9·1	Veronal	0·025	—	0·10	—
A5MP	0·0005	6·5	Succinate	0·050	0·001	—	—
ATP	0·0005	6·5	Succinate	0·050	0·001	—	—
APP	0·0005	6·9	Maleate	0·050	0·001	0·05	—
PP	0·0005	6·9	Glycylglycine	0·10	0·001	—	0·02

protein precipitate used in the Martin & Doty method of inorganic PO_4 estimation. Each tube contained N-H_2SO_4 (2·0 ml.) and 0·02 M-silicotungstic acid (0·5 ml.). The tube was shaken gently to ensure that the section unfolded. The benzene:*iso*butanol (1:1, v/v) mixture (3·0 ml.) and ammonium molybdate (5% w/v; 0·5 ml.) were added. The tube was stoppered and shaken vigorously for 15 sec. Separation of the layers was completed by centrifugation. A portion of the organic solvent layer (2·0 ml.) was added to 2% H_2SO_4 (v/v) in absolute ethanol (2·0 ml.), followed by $SnCl_2$ in N-H_2SO_4 (0·1 ml.). The latter solution was prepared freshly by the 100-fold dilution of the 40% solution of $SnCl_2.2H_2O$ (40 g. in 100 ml. 11 N-HCl). Absorptions at 700 mμ. were measured in a Hilger Uvispek spectrophotometer, using as a blank the extract from the section removed at the start. The results were expressed as μmoles inorganic PO_4 liberated/g. fresh tissue/hr. The proportion of the substrate hydrolysed was always less than 10%.

Alternate sections were stained for visual examination in order to ensure that comparable anatomical structures were present in the series used in any one determination. If the deposit was $Pb_3(PO_4)_2$, the section was washed in four changes of water, treated with dilute ammonium sulphide solution, dehydrated and mounted. If the deposit consisted of calcium phosphate, the section was treated directly with $Co(NO_3)_2$ solution, and then washed, treated with ammonium sulphide solution and mounted.

The conditions used for the incubation of sections are detailed in Table 1. They were those considered to be preferable in previous work on the basis of visual comparisons of substrate specific staining of sections. There appeared to be no activation by the addition of Mg or Ca to the ATP medium. The use of low Pb concentrations reduced the intensity of the diffuse background staining both in experimental and in control sections incubated in the absence of substrate, probably owing to a reduction of the proportion of the initial inorganic PO_4 content (14–16 μmoles/g.) retained in the tissue at the start of incubation. All the media were found to be saturated with respect to inorganic PO_4.

Enzyme activity in fresh tissue. The whole brain (removed as for histological work) was weighed and ground rapidly in 0·89% NaCl at 0°, using a Pyrex glass tube and pestle. The motor was run at 2000 rev./min. for 30 sec. with a pestle diameter of 1·4 cm. The degree of grinding was checked histologically in order to ensure that it was sufficient to disrupt cell bodies. The preparations were diluted with further saline to contain 50 mg./ml. of fresh tissue and stored when necessary at −78°. Incubations were carried out at 37·5° with agitation. The test solution volume was usually 9 ml. so that four samples of 2 ml. could be withdrawn. Proportions of the constituents corresponding to those used in the histological methods were mixed, the solution centrifuged to remove any inorganic PO_4 precipitate and made up to the final volume less that of the tissue suspension. The latter was added as soon as temperature equilibrium was reached. The first sample was withdrawn immediately and 0·02 M-silicotungstic acid (0·5 ml.) added, followed by ammonium molybdate (5% in 4-N H_2SO_4; 0·5 ml.). The subsequent procedure was the same as that used for the estimation of inorganic PO_4 in microtome sections. Since a precipitate of lead or calcium phosphates formed as the reaction proceeded, it was preferable to pipette the other samples into separate tubes soon after the start. The proportion of the substrate hydrolysed was always less than 5%.

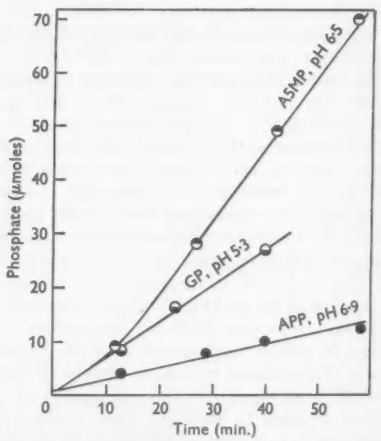

Fig. 1. Rate of accumulation of inorganic phosphate in microtome sections during incubation with adenosine-5-monophosphate, glycerophosphate and aneurin pyrophosphate. Coronal sections at the level of the posterior cerebrum and midbrain. Incubation conditions as in Table 1 at 37·5°.

RESULTS

Enzyme activity in sections. Fig. 1 shows typical determinations of inorganic phosphate in adjacent sections incubated for various times in the same medium. The inorganic phosphate content of the sections increased linearly with time over practically all the range studied. This increase in inorganic phosphate content was accompanied always by a corresponding increase in the intensity of specific staining of the adjacent section incubated on the same slide.

Table 2. *Phosphatase activity in fresh tissue suspensions and histological sections*

(Results are expressed in μmoles P/g. fresh tissue/hr. The levels at which coronal sections were made are indicated as follows: (I) Anterior cerebrum; (II) middle cerebrum; (III) posterior cerebrum and midbrain; (IV) cerebellum and pons; (V) medulla.)

Values from individual experiments

Substrate	pH	Exp. group	Fresh whole brain	Coronal sections
A5MP	6·5	A	106	99 (III)
			94	94 (III)
		B	140	65 (I), 112 (II), 94 (III)
			154	—
		C	82	60 (II), 81 (III), 106 (IV), 105 (V)
			110	—
GP	5·3	A	21	50 (I), 46 (II), 60 (III)
			26	—
			16	—
		B	24	37 (II), 36 (III), 40 (IV), 58 (V)
			31	39 (III), 43 (V)
			28	—
			24	—
GP	9·1	A	26	24 (I), 15 (II), 25 (III)
			22	—
			21	—
		B	26	20 (II), 20 (III), 28 (IV), 18 (V)
			34	46 (III)
APP	6·9	A	11·5	14 (II), 12 (III)
			14·5	18 (I), 11 (II), 13 (III)
		B	8·6	25 (II), 16 (III)
			15·6	23 (II)
			13·0	—
		C	15·1	11 (II), 22 (IV), 15 (V)
			13·4	—
			10·2	—
ATP	6·5	A	41	32 (I), 82 (III)
			71	—
		B	65	91 (I), 37 (II), 47 (III), 40 (IV), 53 (V)
			68	—
			45	—
PP	6·9	—	122	9 (II), 1 (III), 3 (IV)
			130	—

Summary and comparison with the results of other workers

Substrate	pH	Fresh tissue suspension (mean ± S.D.)	Histological sections (mean ± S.D.)	Values from literature*
A5MP	6·5	114·3 ± 25·2	90·7 ± 17·3	58† (Reis, 1937)
GP	5·3	24·3 ± 4·5	45·4 ± 8·5	7·1 (Gordon, 1950)
GP	9·1	25·8 ± 4·7	24·5 ± 9·0	18 (Gordon, 1950)
APP	6·9	12·7 ± 2·3	16·4 ± 4·6	0·7–1·4 (Westenbrink, Steyn Parvé & Goudsmit, 1943)
ATP	6·5	58·0 ± 12·5	54·6 ± 21·2	900 (DuBois & Potter, 1943) 1040‡ (Gore, 1951)
PP	6·9	126	4·3	250 (Gordon, 1950) 1150‡ (Gore, 1951)

* Rat brain except for values marked ‡ which are from guinea pig.
† Inosine-5-phosphoric acid.

Comparison of sections and fresh tissue. The phosphatase activities of frozen, dried, paraffin sections and fresh tissue suspensions are compared in Table 2. Some comparisons were made by dividing the brain into left and right halves, freeze-drying one half and preparing a fresh saline suspension from the other. Otherwise, litter mates or rats of similar ages were included in any one experimental group. Sections were cut coronally at five different anatomical levels. A series of adjacent sections was used in making each determination. The absence of large differences in activities for different anatomical levels was consistent with the presence of stained structures in all sections although the staining was localized. The enzyme activity of the prepared sections was slightly below or of a similar order to that found in the procedure using fresh tissue in those comparisons in which the substrate was A5MP (pH, 6·5), ATP (pH, 6·5), or GP (pH, 9·1). Table 2 shows a considerable overlap in the ranges of the two series of determinations for these substrates. The differences between the means were not significant in relation to the spread of the results. On the other hand, the activity of sections appeared to be rather higher than that obtained with fresh tissue when the substrate was APP (pH, 6·9) or GP (pH, 5·3). The ranges partly overlapped when APP was used. There was no overlap and a significant difference ($P < 0{\cdot}01$) was present when the substrate was GP at pH 5·3.

The hydrolysis of inorganic pyrophosphate (PP) showed a different kind of behaviour. Repeated attempts to detect this activity by a histological method were unsuccessful and the rate of inorganic phosphate deposition in incubated sections was little greater than in the absence of substrate. Nevertheless, fresh tissue suspensions showed high PPase activity under similar conditions.

DISCUSSION

It might be concluded from the work to which reference was made in the introduction that histological methods for the study of phosphatase distribution can demonstrate only a small part of the enzyme activity originally possessed by the tissue. However, it has been found in the present work that the activities in histological sections are, with one exception, close to those shown by fresh tissue suspensions under similar conditions. Probably, a major cause of enzyme loss during tissue preparation has been removed by avoiding chemical fixation, whereas infiltration of the tissue with melted paraffin wax and its removal by light petroleum have only a slight effect on these enzymes. This conclusion might seem to contradict the results of earlier workers (Danielli, 1946; Stafford & Atkinson, 1948; Rabinovitch *et al.* 1949; Berenbom *et al.* 1952) who found considerable phosphatase losses during paraffin infiltration and subsequent treatment. These losses are probably explained by the use of acetone or ethanol to displace the xylene or benzene used to dissolve the paraffin, and are avoided in the procedure of Naidoo & Pratt (1951) since rehydration of the tissue takes place in the incubation medium. Doyle (1948) used carbon disulphide for paraffin removal with only moderate losses (alkaline phosphatase 55 % and acid phosphatase 60 % of the values before embedding), although he used more severe conditions of paraffin infiltration (1 hr. at 60°) than in the present work.

Sensitivity of PPase to the paraffin treatment may explain the absence of appreciable activity in the histological procedure, but attention is drawn to the ease with which PPase can be separated from the tissue in soluble form. The supernatant of a saline suspension of brain after centrifugation contains 80–90 % of the activity, whereas the major part of brain ATPase remains in the tissue residue under similar conditions (Gore, 1951).

The use of the activity of a fresh tissue suspension as a standard with which to compare the values obtained in the histological methods was an arbitrary choice so that undue emphasis should not be placed on the higher values found in the latter method when APP is used as the substrate. The basis of comparison is extended by consideration of some previously reported values for the activities of these enzymes in brain, which are given in the final columns of Table 2. Much higher figures have been reported by previous workers for brain ATPase but not for the other enzymes. The present work suggests that brain ATPase activity is not greatly reduced by freezing, drying and paraffin treatment in the histological procedure. However, there are other special factors which apply equally to histological sections and fresh tissue suspensions. Lead was present in the histological incubation medium and it was used also in fresh tissue suspensions. Solubility considerations restrict the choice of buffer and pH and limit the substrate concentration. Lower activity can be expected under these conditions (ATP, 0·0005 M; no Mg; pH 6·5) since minimum values for maximal activity are ATP, 0·0025 M, Mg 0·0077 M and pH 7·4 (Gore, 1951). It is believed that further investigation of the effects of lead and magnesium will explain the lower ATPase values found in the present work. However, the possibility exists also that the ATPase activity of brain is a complex system and not all the enzymes are represented in the histological results.

The histological methods for 5-nucleotidase, APPase and the phosphatases hydrolysing GP all yield values which are higher than those previously reported by workers using purely biochemical methods. Although inorganic phosphate in solution

in the assay medium may reduce enzyme activity, it is not considered likely that the precipitation of this inorganic phosphate by lead or calcium in the present technique can produce a sufficient effect to account for the increased rates. The largest difference, that for APPase, may be explained by the low substrate concentration, probably much below the optimum, which Westenbrink, Steyn Parvé & Goudsmit (1943) used on account of their analytical method. With GP, previous workers have used acetone in the preparation of enzyme extracts from brain. Such treatment is likely to reduce activity and has been avoided in the present method. Brain acid phosphatase was not found by Giri & Datta (1936) to be activated by magnesium, whereas magnesium addition to the incubation medium has been found advantageous in the present work. For 5-nucleotidase, magnesium addition has not been found desirable, although it was used by Reis (1937). The existence of liver acid phosphatase in a form inactive to GP (Berthet & de Duve, 1951; Berthet, Berthet, Appelmans & de Duve, 1951) does not appear to be important to the present work since all the procedures under consideration seem likely to activate the enzyme. Probably, the relatively higher activities found in some of the histological procedures are due to a failure to obtain the full enzyme activities in the fresh tissue estimations. The enzyme activity represented in five of the histological procedures is of a similar order to or above that estimated by purely biochemical methods so that suitably chosen histological methods can justifiably take a place alongside those more commonly used for enzyme study.

SUMMARY

1. The phosphatase activity in microtome sections has been assessed by the estimation of phosphate deposited in these sections in the presence of lead or calcium and compared with that in fresh tissue suspensions under the conditions of the histological method.

2. The activities of acid and alkaline phosphatases, 5-nucleotidase, adenosine triphosphatase and aneurin pyrophosphatase are of a similar order in frozen, dried, paraffin-infiltrated brain tissue and in fresh tissue. These values are often much greater than previously reported values, probably largely because the tissue was prepared without using acetone or ethanol.

3. It is concluded that the staining obtainable in these methods represents a high proportion of the potential activity of the tissue under the conditions used.

4. Pyrophosphatase activity was negligible in the histological preparations, possibly owing to a high solubility of this enzyme in saline media.

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Studies in Detoxication

51. THE DETERMINATION OF CATECHOLS IN URINE, AND THE FORMATION OF CATECHOLS IN RABBITS RECEIVING HALOGENOBENZENES AND OTHER COMPOUNDS. DIHYDROXYLATION *IN VIVO*

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Naphthalene, anthracene and phenanthrene are metabolized in rats and rabbits to 1:2-dihydroxy compounds which have been proved to be 1:2-dihydro-1:2-diols (Young, 1950; Boyland, 1950). In the benzene series, an *o*-dihydroxy compound, 4-chlorocatechol, is a major metabolite of chlorobenzene in the rabbit (Spencer & Williams, 1950; Smith, Spencer & Williams, 1950). Many monosubstituted benzenes, e.g. phenol and nitrobenzene (cf. Robinson, Smith & Williams, 1951; Garton & Williams, 1949) also form small amounts of catechols. The addition or substitution of two hydroxyl groups ortho to each other, i.e. *o*-dihydroxylation, appears therefore to be a biological reaction of several aromatic compounds, and may result in the formation of reduced diols of the type mentioned above (this reaction has been called 'perhydroxylation') or of catechol derivatives, in which the aromatic ring is not reduced. Whether these two forms of dihydroxylation are related is not known at present.

The objects of the present work were to develop a quantitative method for the determination of catechols in the urine of rabbits receiving benzene derivatives and to find out whether other halogenated benzenes behaved like chlorobenzene.

COLORIMETRIC DETERMINATION OF CATECHOLS

The present method is based on the formation of intensely blue-coloured cobalt complexes of catechol derivatives in the presence of strong alkali. The various complexes of catechol with bivalent cobalt have been described by Weinland & Döttinger (1918). These complexes are stable to alkali. The addition of 10N-sodium hydroxide to cobaltous nitrate solution results in the precipitation ultimately of the brown cobaltic hydroxide, but in the presence of 1:2-dihydroxybenzenes a permanent deep blue colour is obtained instead. A similar blue colour, attributed to the tetrahydroxy cobalt ion $[Co(OH)_4]^{2-}$, is obtained by adding a cobaltous salt to excess saturated aqueous potassium hydroxide or sodium hydroxide (Gordon & Schreyer, 1951). The absorption spectrum of the blue cobalt-catechol coloured complex using 4-chlorocatechol is shown in Fig. 1.

The colour reaction was given by all aromatic compounds tested which contained two or more adjacent hydroxyl groups, e.g. 4-halogeno-, 4-nitro- and 4-amino-catechols, adrenaline and noradrenaline. Pyrogallol and hydroxyquinol gave unstable colours. With monohydric and *m*- and *p*-dihydric phenols no colour was obtained. The colour,

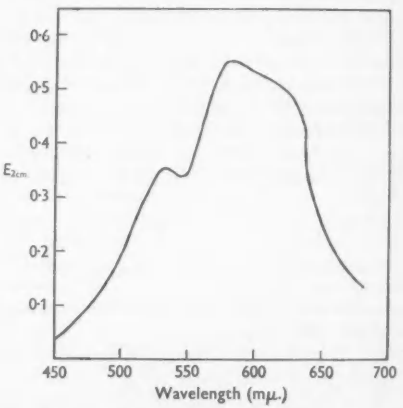

Fig. 1. The absorption spectrum of the blue colour obtained when $Co(NO_3)_2$ reacts with 4-chlorocatechol in the presence of NaOH. Solution prepared by adding 2 ml. of 0·025% aqueous 4-chlorocatechol to 2 ml. of 0·8% aqueous $Co(NO_3)_2.6H_2O$, then adding 4 ml. of 40% NaOH, mixing and centrifuging (see text).

however, is given by glucose, glucuronic acid and tartaric acid since these compounds contain vicinal hydroxyl groups. Glycollic, lactic and uric acids gave very pale colours but urea and furfural did not react. A pale-blue colour which fades completely in 5 min. is also given by phosphoric acid and phosphates.

The effect of cobalt and alkali concentrations. The maximum colour was obtained when 12–20 mg. $Co(NO_3)_2$/8 ml. was

used. The concentration of NaOH was important since it affected the blank solution. It was eventually found that using the optimum amounts of cobalt, a final concentration of 5 N-NaOH was necessary to produce the maximum colour and the least coloured blank. KOH gives much less colour than NaOH (cf. Gordon & Schreyer, 1951) and whereas ammonia solution gave more highly coloured solutions than NaOH, it also produced more highly coloured blanks. Simultaneously with the production of colour there is a slight precipitation of $Co(OH)_3$ which tends to destroy the colour. The blue colour remained stable for more than 1 hr. if this precipitate was removed by centrifuging within 5 min.

Preparation of the standard curve. To a series of 10 × 1 cm. centrifuge tubes, there were added 0·2–2·0 ml. of 4-chlorocatechol solution (25 mg./100 ml. water) and the volume in each tube was made up to 2 ml. with water. Then to each tube 2 ml. of $Co(NO_3)_2$ solution (0·8%, w/v, $Co(NO_3)_2.6H_2O$ in water) and 4 ml. of 40%, w/v, NaOH were added. The contents of the tubes were mixed by twice inverting and the solutions were centrifuged for 5 min. The extinction at 580 mμ. (slit width, 0·5 cm.) of each solution was then measured in 2 cm. cells in the Unicam S.P. 500 spectrophotometer against a blank solution prepared in the same way as the test solution but replacing the chlorocatechol solution by water. The standard curve was linear for concentrations of 4-chlorocatechol up to 30 μg./ml. A similar curve was obtained with catechol, 4-fluoro-, 4-chloro-, 4-bromo-, 4-iodo- and 4:5-dichloro-catechols. The Lambert-Beer Law was obeyed by each compound and the optical density of the cobalt colour was inversely proportional to the molecular weight of each catechol. From pure solutions the recovery of the catechols was 97–98%.

Application of the method to urine. Many substances occur in urine which give a blue colour with $Co(NO_3)_2$ under the conditions described above, notably glucuronic acid which gives a very intense colour. A solution of glucurone, even after heating at 110° for 3 hr. with 3·3 N-HCl, still gives an intense colour. The reaction could not, therefore, be applied directly to urine. However, the catechols involved in the present investigation are ether soluble, whereas the interfering substances are not. Total catechols (i.e. free and conjugated) in urine could, therefore, be readily estimated if the urine was first hydrolysed and the catechols then extracted with ether. Free catechols in urine, however, could not be estimated by this method, since certain conjugates of glucuronic acid which also give a blue colour with $Co(NO_3)_2$, e.g. catechol glucuronides, are soluble in ether.

The recovery of chlorocatechol from urine. The 24 hr. normal urine from a rabbit was filtered and made up to 100 ml. with water. 4-Chlorocatechol (about 5 mg.) was added to 10 ml. of the diluted urine which was then boiled under reflux condenser on a sand bath for 3 hr. with 5 ml. of conc. HCl. Simultaneously a second 10 ml. of diluted urine without added chlorocatechol was treated in the same way, to provide the blank value for normal urine. After cooling, the solutions were continuously extracted with ether for 4 hr. (This period of extraction was determined in separate experiments.) The ether extracts were evaporated and the residues dissolved in water, filtered and made up to 25 ml. The determination of catechol was then carried out as above on 2 ml. of solution. The recovery of 30–50 mg. 4-chlorocatechol/100 ml. was 96–98%. The blank value was usually small and in thirty-two experiments it varied from 0·75 to 7·1 mg./day (average 3·8 mg.) calculated as catechol.

CATECHOL FORMATION FROM VARIOUS BENZENE DERIVATIVES

Materials and methods

Reference compounds. 4-Chlorocatechol, m.p. 80°, and 4:5-dichlorocatechol, m.p. 105°, were prepared according to Peratoner (1898); 4-fluorocatechol, m.p. 83°, according to Corse & Ingraham (1951); 4-bromocatechol, m.p. 85°, according to Rosenmund & Kuhnhenn (1923) and 4-iodocatechol, m.p. 92°, according to Fourneau & Druey (1934). When 4-iodocatechol in aqueous solution is heated with mineral acids, it is deiodinated to yield catechol and this had to be kept in mind during our experiments on iodobenzene. *o-* and *p*-Chloro-, *p*-bromo-, *o-* and *p*-iodo-phenols were purchased (British Drug Houses Ltd.); *o-*, *m-* and *p*-fluoro-, *m*-chloro-, *o-* and *m*-bromo- and *m*-iodo-phenols were prepared by methods quoted in the literature. These compounds were purified by crystallization and chromatography of ethereal solutions on alumina, and m.p.'s and b.p.'s were checked with the literature.

The following new compounds were prepared by standard methods: *4-fluorocatechol ditoluene-p-sulphonate*, m.p. 143°, colourless prisms from ethanol. (Found: C, 55·6; H, 4·1; S, 14·1. $C_{20}H_{17}O_6S_2F$ requires C, 55·1; H, 3·9; S, 14·7%.) *4:5-Dichlorocatechol ditoluene-p-sulphonate*, m.p. 201°, prisms from ethanol. (Found: C, 49·7; H, 3·7; S, 12·7. $C_{20}H_{16}O_6S_2Cl_2$ requires C, 49·3; H, 3·3; S, 13·2%.) *4-Bromocatechol ditoluene-p-sulphonate*, m.p. 134°, prisms from ethanol. (Found: C, 48·1; H, 3·2; S, 12·4. $C_{20}H_{17}O_6S_2Br$ requires C, 48·3; H, 3·4; S, 12·9%.) *4-Iodocatechol ditoluene-p-sulphonate*, m.p. 141°, prisms from ethanol. (Found: C, 44·8; H, 3·2. $C_{20}H_{17}O_6S_2I$ requires C, 44·1; H, 3·1%.) Since the last ester was not easily purified it was more convenient to use *4-iodocatechol dibenzoate*, m.p. 130°, needles from ethanol:ethyl acetate, as a reference compound. (Found: C, 54·1; H, 3·2. $C_{20}H_{13}O_4I$ requires C, 54·1; H, 2·9%.)

Paper chromatography of the monohalogenophenols. Johnson, Stein & Weiss (1951) have shown that the separation by paper chromatography of the isomeric monochlorophenols themselves was not possible. This we can confirm. They, however, achieved separation after coupling the phenols with diazotized sulphanilic acid. This method can be applied to the fluoro-, bromo- and iodo-phenols. Johnson *et al.* (1951) applied their coupled phenols to the paper in *iso*butanol, but in the present work ether was used. Ether solutions or extracts of the phenols (varying quantities up to 5 mg. were used) were evaporated to dryness and the residue coupled at 0° with diazotized sulphanilic acid. The product was extracted with ether. The latter extract was then placed drop by drop at the starting point of the chromatogram (Whatman no. 1 paper) and the ether allowed to evaporate. In this way varying quantities of the coupled phenols could be used. The chromatograms were run in the solvent described by Johnson *et al.* (1951). With small quantities of the phenols, the *o*-isomers gave two spots, the *p-* and *m-* one spot each as described by Johnson *et al.* for the chlorophenols. With larger quantities of phenols, the *o*-phenols gave up to five spots, the *p-* four spots and the *m*-phenols usually one but very occasionally two spots. With the bromophenols, for example, the R_F values of the spots in a chromatogram run for 16 hr. were: ortho, 0·24 (orange-yellow), 0·39, 0·58, 0·70 (yellow) and 0·75 (blue); meta, 0·33 (orange-yellow); para, 0·63 (yellow), 0·69, 0·78 and 0·84 (orange-yellow). All the *o*-phenols gave the fast-running

blue spot when they were used in larger quantities. Several control tests were carried out to eliminate possible artifacts. With the *o*- and *p*-phenols the spots were not of the same intensity, since the number which appeared depended on the amount of coupled phenol put on the paper initially. We cannot explain the multiplicity of spots from the authentic *o*- and *p*-phenols which were purified by chromatography, except to suggest, as did Johnson *et al.* (1951), that they may be due to coupling in more than one position at the same time and separately. The *o*- and *p*-phenols were thus readily distinguished from the *m*-phenols. A very rough idea of the amounts of *o*- and *p*-phenols could be made from the size and number of the spots formed.

The urines after feeding the fluoro-, chloro- and bromobenzenes were hydrolysed with 0·5 vol. conc. HCl for 3 hr. In the case of iodobenzene the urine only received mild acid hydrolysis to avoid possible de-iodination of iodophenols by the stronger acid. The hydrolysed urines were then exhaustively extracted with ether, and the extracts evaporated to dryness at room temperature to avoid loss of volatile phenols. The residues were extracted with cold water to remove catechols and the insoluble material was redissolved in ether, filtered and evaporated as before. The residue was then coupled at 0° with diazotized sulphanilic acid, the product extracted with ether and the extract chromatographed as above.

Animals. Chinchilla doe rabbits (2–3 kg. wt.) were kept on a constant diet of 70 g. rat cubes/day ('Diet 41'; Associated London Flour Millers Ltd.). All compounds were administered orally, and the urine from each animal was collected and analysed daily for catechol as above.

RESULTS

The results of the quantitative experiments are given in Table 1. Qualitative tests showed that the catechols were excreted mainly in the conjugated form (cf. Smith *et al.* 1950). Fig. 2 shows the rate of elimination of catechols after single doses of halogenobenzenes. Up to 4 days may be necessary before catechol excretion ceases, although the bulk is excreted on the first 2 days. The peak excretion of catechols occurred most frequently on the first day but sometimes on the second day after feeding. In the case of *o*-dichlorobenzene, the peak excretion always occurred on the second day; the fate of the dichlorobenzenes, however, will be discussed in a future paper. With the phenols, increased catechol excretion only occurred for one day after dosing, and never on subsequent days.

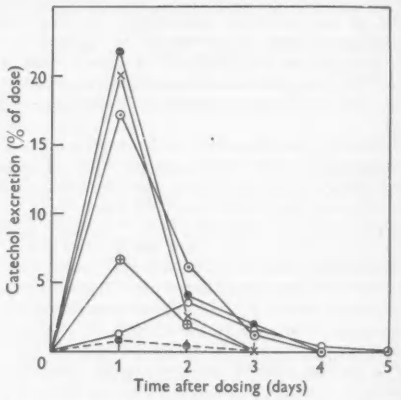

Fig. 2. The excretion of catechols by rabbit no. 17 after dosing with benzene and halogenobenzenes. ●- - -●, Benzene; ●—●, bromobenzene; ×—×, iodobenzene (rabbit no. 21); ⊙—⊙, chlorobenzene; ⊕—⊕, fluorobenzene; ○—○, *o*-dichlorobenzene.

Qualitative experiments

General treatment of urines. The urines were collected for 48 hr. The free phenols were removed from the urines by continuous extraction with ether for 10 hr. at pH 5. Phenols present as ethereal sulphates were similarly extracted with ether for 12 hr., after the residual urine had been made 0·5 N with respect to HCl and heated for 0·5 hr. at 100°. The phenols present as glucuronides were similarly extracted after the residual urine had been made 3 N with respect to HCl and boiled for 3 hr. at 100°. Each ethereal extract was dried over anhydrous Na_2SO_4 and after removal of the ether, the gummy residues were further fractionated into fractions soluble in cold water (mainly catechols) and soluble in hot water (monophenols and mercapturic acids).

Table 1. *Excretion of catechols by rabbits receiving oral doses* (0·5 *g./kg.*) *of halogenobenzenes and chlorophenols*

Compound fed	Period of excretion of catechols (days)	Catechol excretion (% of dose)			Av.
Benzene	2	1·7	2·5	3·2	2·5
Fluorobenzene	2	8·8	12·8	13·7	11·8
Chlorobenzene	3	24·7	27·8	28·8	27·1
Bromobenzene	4	27·9	28·0	28·7	28·2
Iodobenzene	2	18·2	21·3	22·9	20·8
o-Dichlorobenzene	3–4	7·0	7·7	8·6	7·8
p-Chlorophenol	1	1·4	1·7	3·2	2·1
m-Chlorophenol	1	4·4	4·7	—	4·5
o-Chlorophenol	1	1·3*	1·5*	1·7*	1·5
Phenol	1	0·4*	0·5†	0·8*	0·6

* Dose 0·3 g./kg. † Dose 60 mg./kg.

Fluorobenzene. The free-phenol fraction from the urine of three rabbits which had received collectively 4 g. of fluorobenzene weighed 150 mg. Colour tests showed that it contained no fluorocatechol (e.g. no green colour with $FeCl_3$), but probably some monofluorophenols (blue colour with 2:6-dichloroquinone chloroimide at pH 8), but crystalline compounds were not isolated. The ethereal sulphate fraction (180 mg.) yielded, after esterification with toluene-*p*-sulphonyl chloride, 4-fluorocatechol ditoluene-*p*-sulphonate (10 mg.), m.p. and mixed m.p. 143°. The glucuronide fraction (1·6 g.) also yielded the same ditoluene-*p*-sulphonate (65 mg.), m.p. and mixed m.p. 142–143°. (Found: C, 55·9; H, 4·1%.)

Bromobenzene. From the urine of a rabbit which had received 3 g. of bromobenzene, the free-phenol fraction (100 mg.) yielded 64 mg. of material soluble in cold water containing 4-bromocatechol. A few mg. of 4-bromocatechol ditoluene-*p*-sulphonate (m.p. 128°) were isolated. The fraction insoluble in cold water also yielded a few mg. of *p*-bromophenylmercapturic acid, m.p. 148° (authentic m.p. 153°) and gave colour tests for monobromophenols none of which were crystallized. The part of the ethereal sulphate fraction soluble in cold water (720 mg.) was precipitated with basic lead acetate at pH 9. Removal of the lead with H_2S yielded a solution of 4-bromocatechol from which 298 mg. (3·1% of dose) of the ditoluene-*p*-sulphonate was isolated, m.p. and mixed m.p. 134°. *p*-Bromophenylmercapturic acid (35 mg., m.p. 148°, mixed m.p. 149°) was isolated from the part insoluble in cold water. The glucuronide fraction (1·225 g.) also yielded 4-bromocatechol ditoluene-*p*-sulphonate (310 mg. or 3·2% of dose), m.p. and mixed m.p. 134° (Found: C, 48·6; H, 3·8%); and *p*-bromophenylmercapturic acid (25 mg.), m.p. and mixed m.p. 150°. Monobromophenols were present in all fractions but none could be isolated.

Iodobenzene. Iodobenzene (4 g.) was fed to a rabbit and a 96 hr. urine collected. The urine gave a deep-green colour with $FeCl_3$ and a strong naphthoresorcinol test. The free phenol fraction (930 mg.) yielded no crystalline material, but colour tests indicated the presence of a catechol derivative (green colour with $FeCl_3$) and of *p*-iodophenol (blue colour slowly developing at pH 10 with 2:6-dichloroquinone chloroimide after removal of the catechol with basic lead acetate). The ethereal sulphate fraction (4 g.) gave a strongly positive naphthoresorcinol reaction as well as a green catechol reaction with $FeCl_3$. An ether-soluble glucuronide had thus been extracted. The part of this fraction soluble in cold water was extracted with $CHCl_3$ and from this 590 mg. (5·5% of dose) of 4-iodocatechol ditoluene-*p*-sulphonate, m.p. and mixed m.p. 141° (Found: C, 44·6; H, 3·2%) was isolated after esterification. 4-Iodocatechol was also identified as the dibenzoate, m.p. and mixed m.p. 130°. (Found: C, 54·4; H, 3·0%.) No other crystalline compound was isolated from this fraction.

The glucuronide fraction contained free iodine (equivalent to 5·5% of dose) which was probably derived from 4-iodocatechol during the hydrolysis in strongly acidic solution since the 600 mg. of this fraction soluble in cold water yielded, after lead-acetate precipitation and esterification, catechol ditoluene-*p*-sulphonate, m.p. and mixed m.p. with an authentic sample (m.p. 162–163°, Porteous & Williams, 1949) 162° (yield 240 mg. or 2·9% of dose).

p-Iodophenylmercapturic acid was isolated from the 2-day urine of three rabbits which had received 2 g. each of iodobenzene. The urine was made N with respect to HCl and extracted three times with 0·2 vol. of $CHCl_3$. The mercapturic acid was then transferred to 2N-$NaHCO_3$ and the bicarbonate extract washed once with ether. The solution was acidified with HCl and the mercapturic acid extracted with $CHCl_3$ which on evaporation yielded 187 mg. of the acid (1·9% of dose). On recrystallization from water (charcoal) 66 mg. of pure *p*-iodophenylmercapturic acid were obtained, m.p. 154° and $[\alpha]_D^{22} - 18° \pm 2°$ in ethanol (*c*, 1). (Found: C, 36·6; H, 3·5; SH after alkaline hydrolysis, 8·9. Calc. for $C_{11}H_{12}O_3NSI$; C, 36·2; H, 3·3; SH, 9·0%.) Parrod (1948) records m.p. 150° and $[\alpha]_{5461} - 11·8°$ and Baumann & Schmitz (1895) m.p. 152–153° and $[\alpha]_D - 10·7°$ in ethanol (*c*, 2·5).

o-Dichlorobenzene. Three rabbits were each fed with 1·5 g. of *o*-dichlorobenzene and their urines collected for 24 hr. The pooled urines were boiled under reflux condenser for 3 hr. with 0·5 vol. of conc. HCl and the resulting solution exhaustively extracted with ether. The phenols in the ether were transferred to 20 ml. of 2N-NaOH and the alkaline solution then shaken with 1 g. of toluene-*p*-sulphonyl chloride in 20 ml. of acetone. On dilution with water and fractional crystallization of the product from ethanol, there was obtained 4:5-dichlorocatechol ditoluene-*p*-sulphonate (180 mg.), m.p. and mixed m.p. 201° (Found: C, 49·1; H, 3·6%), and an unidentified ester (260 mg.), m.p. 98°.

That *p*-chlorophenol is a minor metabolite of chlorobenzene was shown by Smith *et al.* (1950) by isolation. This has now been confirmed by paper chromatography and it has also been shown that the other three monohalogenobenzenes form the corresponding *p*-halogenophenols. It was shown (p. 147) that the *o*-phenols, after coupling with diazotized sulphanilic acid, produce a fast-moving blue spot, whereas the *m*- and *p*-isomers do not. This spot was obtained with all halogenobenzene urines when the extracts were used in appropriate amounts. As far as the chromatographic method goes it appears that *o*-phenols are formed in very small amounts. No *m*-halogenophenols were detected in any of the four urines. By comparing the size and number of spots obtained from urine extracts with those obtained with the authentic phenols it was possible to make a very rough estimate of the amounts of phenols present. This was about 2–3% of the dose for the *p*-isomer and not more than about 0·1% for the *o*-isomer.

DISCUSSION

Chloro-, bromo- and iodo-benzenes each form in rabbits two major metabolites, namely the *p*-halogenomercapturic acid and the 4-halogenocatechol. They therefore differ from most other monosubstituted benzenes such as aniline, acetanilide, nitrobenzene, phenol and cyanobenzene which yield little or no mercapturic acid and only small amounts of the 4-substituted catechols. It was earlier suggested (Smith *et al.* 1950) that the formation of catechols may be connected with mercapturic acid formation. In fact, compounds which are *o*-dihydroxylated *in vivo*, e.g. naphthalene, anthracene, phenanthrene and chloro-, bromo- and iodo-benzenes also give rise to much mercapturic acid. In the case of the three halogenobenzenes mentioned above, catechol and mercapturic acid

formation each account for about 20–30 % of the dose metabolized (Table 2). With *o*-dichlorobenzene there again appears to be a correlation between these two types of metabolites, although each process only accounts for 8 % of the dose. The phenols in Table 1 do not form mercapturic acids and only form small amounts of catechols. However, fluorobenzene differs from all other monosubstituted benzenes in that it forms very little mercapturic acid (3 %), yet it is appreciably *o*-dihydroxylated (about 20 %, Table 2). This suggests that there is no correlation between mercapturic acid formation and *o*-dihydroxylation.

Table 2. *A comparison of the catechol and mercapturic acid outputs of rabbits dosed with various benzene derivatives*

(Values are expressed as percentage of the dose after correction for the amount of the compound which is eliminated unchanged in the expired air. The percentages of the dose eliminated in this way are benzene, 39; fluorobenzene, 44; chlorobenzene, 27; bromobenzene, 6; and iodobenzene, 3 (see Azouz, Parke & Williams, 1952). Data for mercapturic acid excretion are from the same paper.)

	Percentage of dose (corrected) excreted	
Compound fed	As catechol derivatives	As mercapturic acids
Benzene	4·1	1·3
Fluorobenzene	21	3
Chlorobenzene	37	28
Bromobenzene	30	22
Iodobenzene	21	23
o-Dichlorobenzene	8	8*
Phenol	0·5	0†

* Determined iodometrically by Stekol's (1936) method.
† Callow & Hele (1926).

The mechanism of these hydroxylations is at present unknown, but several hypotheses have been put forward (cf. Boyland, 1950). Boyland has suggested that simple addition of oxygen to a double bond to form an epoxide might be the initial reaction. Addition of water to the epoxide could yield a *trans*-dihydrodiol. The latter could then yield a monophenol by dehydration and a catechol by dehydrogenation. Several observations seem to support these possibilities. Epoxides are known to occur naturally, particularly in the carotenoids (cf. Goodwin, 1952) and, recently, heptachlor epoxide has been found as a metabolite of the insecticide heptachlor (a heptachlorotetrahydromethaneindene) in dogs (Radomski & Davidow, 1952). Furthermore, the known natural dihydrodiols of naphthalene, anthracene and phenanthrene (and probably the 3:4-dihydro-3:4-dihydroxychlorobenzene of Smith *et al.* 1950) are *trans*-compounds which would be expected if they were formed by hydration of an epoxide. Dihydrodiols are also readily dehydrated by acids to monophenols whose orientations agree with those found biologically, although Boyland & Wiltshire (1952) did not observe the conversion of 1:2-dihydronaphthalene-1:2-diol to 1-naphthol *in vivo*. Dehydrogenation of dihydrodiols to catechols, however, has not yet been observed chemically or biologically.

In earlier papers from this laboratory (Smith & Williams, 1950; Robinson *et al.* 1951; Smith, 1950) attention was drawn to the similarities between the orientation of biological and of free-radical hydroxylation of benzene derivatives. Recently, Johnson *et al.* (1951) have submitted chlorobenzene to the action of free hydroxyl radicals and found that *o*-, *m*- and *p*-chlorophenols were produced. These results appear to be different from the biological hydroxylation of chlorobenzene where the phenols produced are 4-chlorocatechol, *p*-chlorophenol and *o*-chlorophenol roughly in the ratio of 200–300 : 30 : 1. However, 4-chlorocatechol could be a hydroxylation product of both *m*- and *p*-chlorophenols. *p*-Chlorophenol does form 4-chlorocatechol (Spencer & Williams, 1950). The *m*-phenol probably does so also, but this has not been proved. The *m*-compound yields 2 to 3 times as much catechol as does the *p*-compound (see Table 1). The chlorophenols, however, are rapidly converted to glucuronides and ethereal sulphates (Spencer & Williams, 1950) which are readily excreted and are not likely to be hydroxylated to any large extent (cf. Bray, Humphris, Thorpe, White & Wood, 1952). These phenols may not reach, except in small quantities, the site of hydroxylation which may well be different from that of conjugation. However, the fact that, apart from conjugation reactions, these phenols are converted into catechols could indicate that the catechols are further hydroxylation products of *m*- and *p*-halogenophenols produced by initial hydroxylation of halogenobenzenes. This second hydroxylation would have to take place before conjugation of the monophenol, since conjugates once formed are not further metabolized (Garton & Williams, 1949). For this to happen, however, different sites for hydroxylation and conjugation must be postulated. On these grounds the phenolic metabolites of the halogenobenzenes could be formed by a mechanism similar to free-radical hydroxylation.

SUMMARY

1. A spectrophotometric method for the determination of ether-soluble catechols in urine has been studied. The method depends on the measurement at 580 mμ. of the blue colour formed between cobalt salts and catechol in the presence of alkali. The colour is given by many 1:2-dihydroxy compounds, including adrenaline.

2. This method has been applied to the determination of catechols formed during the metabolism of fluoro-, chloro-, bromo-, iodo- and *o*-dichlorobenzene in the rabbit. Between 20 and 30 % of the monohalogenobenzenes metabolized are converted into the corresponding 4-halogenocatechols which are excreted mainly in conjugated forms for 2–3 days after dosing. Some 8 % of *o*-dichlorobenzene is dihydroxylated. The isomeric monochlorophenols and phenol are also oxidized to catechols to a small extent (0·5–4 %).

3. The halogenobenzenes also form small amounts (about 2–3 %) of *p*-halogenophenols and traces of the *o*-phenols. These were detected mainly by paper chromatography.

4. The relation between mercapturic acid and *o*-dihydroxylation in these compounds is discussed, but no definite conclusions have been reached.

5. It is shown that the formation of catechols from the halogenobenzenes need not be contrary to the free radical hypothesis of biological hydroxylation, although other explanations are also feasible.

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Studies in Detoxication

52. THE APPARENT DISSOCIATION CONSTANTS OF SOME GLUCURONIDES, MERCAPTURIC ACIDS AND RELATED COMPOUNDS

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Quick (1932) noted that the conjugated products formed during detoxication processes in the body were highly acidic, and suggested that the purpose of conjugation may not primarily be a detoxication mechanism in the sense of rendering a toxic group inert, but a means of converting a weak acid into a strong acid which can be readily eliminated. That conjugated compounds such as glucuronides, hippuric acids, arylsulphuric acids are strongly acidic seems to be generally true but the ionization constants of only a few of these compounds have been measured (cf. Williams, 1947).

EXPERIMENTAL

Compounds. The glucuronides, mercapturic acids and phenylacetylglycine used had been prepared biologically from time to time in this laboratory. Their melting points and rotations were checked with the literature. The hippuric acids and ornithuric acid used were synthetic.

Measurement of pK_a. The apparent dissociation constants of the acids at 22° were determined in the usual manner from the potentiometric titration curves. Measurements of pH were made with a Cambridge pH meter using a glass electrode. The compound (15–30 mg.), dissolved in freshly boiled distilled water to give an approximately 0·001 M

solution, was titrated with 0·02 N-NaOH, measuring the pH at 0·1–0·2 ml. intervals. pK_a was taken as the pH at which half the acid was neutralized. Salts were titrated with 0·02 N-HCl.

The pK_a values of amphoteric compounds, such as the aminophenylglucuronides and quinolylglucuronides, were determined by titration with both acid and alkali. With most of the compounds the pK_a values of the acidic and basic groups were often not more than two pH units apart and it was not easy to distinguish between them. The basic pK_a values were, therefore, determined independently by a spectroscopic method (cf. Morton & Lunn, 1952). The ultraviolet-absorption spectra of the aminophenylglucuronides, *p*-aminohippuric acid and quinolylglucuronides, vary with pH. This variation appears to depend on the presence or absence of a positively charged N. The absorption spectra of these compounds were, therefore, measured in 0·1 N-HCl and 0·1 N-NaOH and in buffers of known pH, using a Unicam S.P. 500 spectrophotometer. The pK_a of the basic group was then calculated from the relation

$$pK_a = pH - \log (\epsilon_a - \epsilon)/(\epsilon - \epsilon_b),$$

where ϵ is the molecular extinction coefficient at a given wavelength at a fixed pH, and ϵ_a and ϵ_b are the corresponding extinctions in acid and alkali. The relevant portions of the absorption curves for *p*-aminophenylglucuronide are shown in Fig. 1. The values of the spectroscopically determined $pK_{NH_3^+}$ given in Tables 2 and 3 are the averages of a number of determinations at different pH values. Conjugation has little effect on the $pK_{NH_3^+}$ of the precursor (see Table 2). The pK values for thirty-one conjugated compounds are given in Tables 1–3.

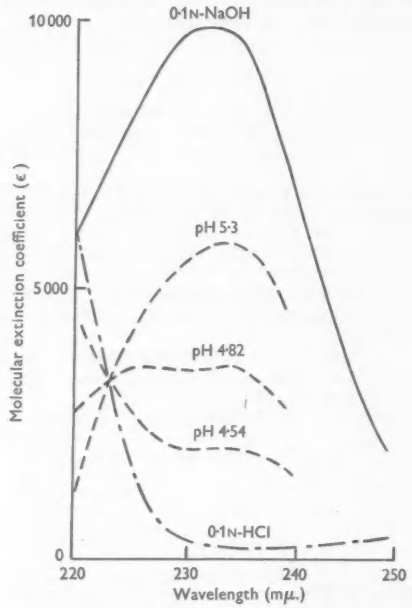

Fig. 1. The ultraviolet-absorption spectrum of *p*-aminophenylglucuronide between 220 and 250 mμ. in 0·1 N-NaOH, 0·1 N-HCl, and acetate buffers of various pH values.

Maximum values of λ and ϵ for the aminophenylglucuronides in acid and alkali are quoted in Table 4. The alkaline spectra of these glucuronides are very similar to the corresponding aminophenols (cf. Morton & Stubbs, 1940).

The optical rotations of the aminophenylglucuronides. The optical rotations of the aminophenylglucuronides in 0·1 N-HCl were determined by Williams (1943). Bray, Clowes & Thorpe (1952*a*) have recently prepared these compounds and determined their rotations in water. It is well known that the rotations of amino acids vary with pH (cf. Neuberger, 1948), and it appears that the aminophenylglucuronides behave somewhat similarly. In acid solutions these glucuronides will occur as cations and in alkali as anions. Table 5 shows that the optical rotations of these compounds are more negative in alkali than in acid, especially those of the *o*- and *m*-isomers. The *p*-isomer, however, only shows very small differences, and Williams (1943) showed that this compound analysed as a monohydrate, whereas the *o*- and *m*-isomers were anhydrous. The sample of the *p*-isomer used in this work was newly prepared and had m.p. 215° (decomp.). (Found: C, 47·6; H, 5·5. Calc. for $C_{12}H_{15}O_7N$, H_2O: C, 47·5; H, 5·6%.) On heating to 120° it lost no water, but on prolonged heating it darkened and showed signs of decomposition.

DISCUSSION

It is clear from Tables 1 and 2 that conjugated glucuronic acids are all fairly strong organic acids having pK_a values between 3 and 4. (Acetic and benzoic acids have pK_a values of 4·7 and 4·2.) It is to be expected, from structural considerations, that the carboxyl group of β-glucuronides would not be very different in acidity from that of glucuronic acid itself, which has a pK_a of 2·93. At the pH of blood (7·4), glucuronides are, therefore, practically completely ionized and, in general, one would expect them to be readily excreted. Furthermore, they are usually stronger acids than their precursors even though these may be carboxylic acids such as veratric acid (pK_a, 4·44). Veratroylglucuronide has pK_a 3·47 (Table 1). Benzoic acid (pK_a, 4·2) forms benzoylglucuronide for which Quick (1932) quotes a pK_a value of about 2·52, which is probably somewhat low. Glucuronides may be formed from primary, secondary and tertiary alcohols in which the pK_a of the hydroxyl group is in the range 12–19, from phenols, pK_a range 5–11, and from some carboxylic acids, pK_a range 3–5. If the purpose of the conjugation is to increase acid strength so as to produce a highly ionizable compound which would be rapidly excreted, it may be expected that, other things being equal, a compound with a pK_a of about 3 or less, i.e. less than that of a glucuronide, would not be conjugated. This is true to some extent since *o*-nitrobenzoic acid (pK_a, 2·21) and *o*-chlorobenzoic acid (pK_a, 2·89) are excreted largely unchanged by rabbits (Bray, Thorpe & Wood, 1949; Bray, Clowes,

Table 1. *Apparent dissociation constants of biosynthetic glucuronides*

(The pK_a values for the precursors are taken from the literature. Alcohols are very weak acids having values between 16 and 19 on an arbitrary pK_a scale, e.g. menthol has a pK_a of 19 (cf. Hammett, 1940).)

β-Glucuronide	pK_a	Precursor	pK_a
(Glucuronic acid)	(2·93)	—	—
Ether type ($R.O.C_6H_9O_6$)			
Phenyl	3·42	Phenol	10
p-Bromophenyl	3·02	*p*-Bromophenol	9·8
p-Iodophenyl	3·28	*p*-Iodophenol	9·5
p-Cyanophenyl	3·22	*p*-Cyanophenol	8·2
p-Diphenylyl	3·10	*p*-Hydroxydiphenyl	—
2:4-Dimethylpentyl-3-	3·89	2:4-Dimethylpentane-3-ol	Approx. 18
(+)-*iso*Menthyl	3·70	(+)-*iso*Menthol	Approx. 18
1-Hydroxyxanthone-7- (Euxanthic acid)	3·94	1:7-Dihydroxyxanthone (Euxanthone)	—
Ester type ($R'.CO.O.C_6H_9O_6$)			
Veratroyl	3·47	Veratric acid	4·44
2-Ethylhexanoyl	3·08	2-Ethylhexanoic acid	—
Diethylacetyl	3·37	Diethylacetic acid	4·7

Table 2. *Apparent dissociation constants of biosynthetic amphoteric glucuronides*

(Values in parentheses were determined spectroscopically. Values for the precursors are taken from the literature. The glucuronides of the hydroxyquinolines are described by Smith (1953). The 2- and 4-quinolones do not conjugate directly but are first hydroxylated, so that *x* may be 3, 5, 6, 7 or 8.)

β-Glucuronide	pK_{COOH}	$pK_{NH_3^+}$	Precursor	pK_{OH}	$pK_{NH_3^+}$
o-Aminophenyl	3·30	4·78	*o*-Aminophenol	9·72	4·72
m-Aminophenyl	2·97	4·44 (4·48)	*m*-Aminophenol	9·89	4·17
p-Aminophenyl	3·1	5·3 (5·2)	*p*-Aminophenol	10·30	5·49
Quinolyl-3-	3·0	3·95	3-Quinolinol	—	—
Quinolyl-5-	3·1	4·91	5-Quinolinol	—	—
Quinolyl-6-	3·0	5·00	6-Quinolinol	—	—
Quinolyl-7-	3·0	5·40 (5·25)	7-Quinolinol	—	—
Quinolyl-8-	3·01	4·85 (4·4)	8-Quinolinol	9·45*	5·03*
2-Quinolone-*x*-	3·07	3·75	2-Quinolone	—	—
4-Quinolone-*x*-	3·15	3·7	4-Quinolone	—	—

* Albert & Magrath (1947).

Table 3. *Apparent dissociation constants of mercapturic, hippuric and ornithuric acids*

(Values for the precursors and acetylglycine are taken from the literature. The $pK_{NH_3^+}$ value 3·19 for *p*-aminohippuric acid was determined spectroscopically.)

Compound	pK_a	Precursor	pK_a
L-Phenylmercapturic acid	3·60	Benzene	—
L-*p*-Chlorophenylmercapturic acid	3·77	Chlorobenzene	—
L-*p*-Bromophenylmercapturic acid	3·81	Bromobenzene	—
L-*p*-Iodophenylmercapturic acid	3·78	Iodobenzene	—
Acetylglycine	3·6	—	—
Hippuric acid	3·70	Benzoic acid	4·2
o-Chlorohippuric acid	3·79	*o*-Chlorobenzoic acid	2·89
o-Hydroxyhippuric acid (Salicyluric acid)	3·62	Salicylic acid	3·23
p-Aminohippuric	4·03 ($pK_{NH_3^+}$, 3·03) 3·19	*p*-Aminobenzoic acid	4·86 ($pK_{NH_3^+}$, 2·32)
Phenylacetylglycine (Phenaceturic acid)	3·67	Phenylacetic acid	4·25
DL-*NN'*-Dibenzoylornithine (DL-Ornithuric acid)	3·70	Benzoic acid	4·2

Table 4. *Maximum values of λ and ε for the aminophenylglucuronides*

	In 0·1 N-HCl		In 0·1 N-NaOH			
Isomer	$\lambda_{max.}$ (mμ.)	$\epsilon_{max.}$	$\lambda_{max.}$ (mμ.)	$\epsilon_{max.}$	$\lambda_{max.}$ (mμ.)	$\epsilon_{max.}$
Ortho	270	890	230	8100	280	2150
Meta	265	1070	232	8600	280	2250
Para	270	750	232·5	9800	290	1800

Thorpe, White & Wood, 1952*b*). Furthermore, *m*- and *p*-nitrobenzoic acids (pK_a 3·46 and 3·4) are also excreted mainly unchanged (Bray *et al.* 1949). However, some strong acids are conjugated (see below).

Table 5. *The optical rotations of the aminophenylglucuronides*

	$[\alpha]_D^{20}$ in		
Isomer	0·1 N-HCl (*c*, 1)	0·1 N-NaOH (*c*, 1)	Water (*c*, 0·5)
Ortho	−77·5°	−94°	−77°
	(−76·1)*		(−87)†
Meta	−94	−106	−97
	(−94)*		(−100)†
Para	−84	−88	−86
	(−82·7)*		(−93)†

* Williams (1943). † Bray *et al.* (1952*a*).

The mercapturic acids quoted in Table 3 all have pK_a about 3·7 and are derived from non-acidic hydrocarbons. The hippuric acids (Table 3) also have pK_a values in the region of 3·7, close, as would be expected, to that of acetylglycine (pK_a, 3·6). Ornithuric acid, which appears to be formed only in birds, also has a pK_a of 3·7. Thus, all the thirty-one conjugated compounds recorded in this paper have pK_a values between 3 and 4. Conjugated sulphuric acids (ethereal sulphates) are even stronger than this, for Burkhardt, Ford & Singleton (1936) have shown that potassium phenylsulphate and potassium *p*-nitrophenylsulphate are salts of strong acids, since they show, with hydrochloric acid, no buffering action detectable by indicators, whereas potassium sulphate shows marked buffering action under the same conditions. Furthermore, in some animals, pyridine (pK_a, 5·21) and quinoline (pK_a, 4·94) are methylated and excreted as the highly ionized methyl-pyridinium and methyl-quinolinium hydroxides which are known to be strong bases, although no pK_a values appear to have been recorded. Furthermore, the weak hydrocyanic acid (pK_a, 8·88) is converted in animals into the much stronger thiocyanic acid.

o-Chlorobenzoic acid (pK_a, 2·89) is excreted largely unchanged by rabbits, but a small amount of it forms an ester glucuronide which presumably would have a pK_a of just over 3. It also conjugates to a slight extent with glycine (Bray *et al.* 1949) to form *o*-chlorohippuric acid (pK_a, 3·79). Thus, both glucuronic acid and glycine conjugations produce acids weaker than the precursor. It is possible that in order to become conjugated a compound must enter a cell, and owing to its low pK_a, *o*-chlorobenzoic acid only does so to a small extent. Thus its conjugation is low. In the case of salicylic acid (pK_a, 3·23) the formation of salicyluric acid (pK_a, 3·62), or of salicylglucuronides (which would probably have pK_a values about 3–3·5) or gentisic acid (pK_a, 3·32), hardly makes any difference in acidity. However, despite its relatively high acidity, salicylic acid appears to be appreciably oxidized and conjugated in some species. According to Bray, Ryman & Thorpe (1948) nearly 30% may form glucuronides and at least 5% may be oxidized to gentisic acid in the rabbit. In man, it is mainly converted to salicyluric acid (Kapp & Coburn, 1942); some 25% is converted to glucuronides and 4–8% oxidized to gentisic acid. According to Alpen, Mandel, Rodwell & Smith (1951) (cf. also Schayer, 1950) the amounts of metabolites of salicylic acid excreted by man are variable. They give salicylic acid 10–85, salicyluric acid 0–50, gentisic acid up to 1 and salicylglucuronides 15–40% of the dose. Salicylic acid, however, appears to penetrate into all the tissues of the body (Goodman & Gilman, 1941; cf. also Gross & Greenberg, 1948). These facts suggest that compounds may conjugate with glucuronic acid (or glycine in the case of carboxylic acids) irrespective of their pK_a if they can penetrate cells.

According to Höber (1946) a conjugation mechanism may have two effects. In the first place, the toxic substances, which are often weak electrolytes and lipoid-soluble and thus are apt to enter cells, are converted into strong electrolytes and this results in an incapacity to enter cells. The second effect is the formation of a hydrophobic-hydrophilic compound which can be eliminated by way of the kidney. Sperber (1948) has shown that a number of glucuronides and ethereal sulphates and hippuric acid are readily excreted by the tubules in the chicken. The present results show that all conjugates are strong electrolytes, and it is also known that they possess the polar-non-polar or so-called organophilic-hydrophilic structure. Many studies in the past have suggested that the polarity of a molecule largely determined whether or not a compound was

actively excreted by the renal tubules. The relation between pK_a and excretion has been studied by Fisher, Troast, Waterhouse & Shannon (1943) who found that although in many cases the polarity of the compound was important, several weakly polar compounds, e.g. sulphanilylsulphanilamide, were actively secreted whilst some strongly polar molecules, e.g. sulphanilic acid, were not; they concluded that structure and possibly other factors, as well as pK_a, were involved.

The present work shows that the majority of conjugation processes result in the production of relatively strong electrolytes, which are easily excreted, from weaker ones. Organic compounds which are stronger electrolytes than their possible corresponding conjugates tend to be excreted largely unchanged, unless they can penetrate cells. In the latter event conjugation appears to take place irrespective of whether the pK_a of the precursor is greater or less than that of the conjugate formed.

The relation of pK_a to type of conjugation may be summarized: hippuric acid formation, when it occurs, appears to be limited to carboxylic acids where the pK_a range is 2–5, ethereal sulphate formation occurs usually with phenols where the pK_a range is 6–11; glucuronic acid conjugation, however, when it occurs, appears to take place irrespective of pK_a from 2–20.

SUMMARY

1. The apparent dissociation constants of twenty-one biosynthetic β-glucuronides have been measured. The pK_a values were all between 3 and 4, and close to that of glucuronic acid (2·93).

2. *p*-Chloro-, *p*-bromo- and *p*-iodo-phenylmercapturic acids had pK_a values about 3·8, near to that of phenylmercapturic acid (3·6). Ornithuric acid had pK_a 3·7.

3. Five hippuric acids had pK_a about 3·7, close to that of acetylglycine (3·6).

4. The above results are discussed and it is shown that most conjugated compounds are stronger electrolytes than their precursors. Some exceptions to this rule are explained.

5. The absorption spectra and optical rotations of the aminophenylglucuronides have been determined and discussed.

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Studies in Detoxication

53. THE GLUCURONIC ACID CONJUGATION OF HYDROXYQUINOLINES AND HYDROXYPYRIDINES IN THE RABBIT

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Investigations in progress in this laboratory on the metabolism of quinoline necessitated the preparation, as reference compounds, of the glucuronides of all the isomeric monohydroxyquinolines. The possibility was also kept in mind that some of the glucuronides might also find use as substrates for the study of β-glucuronidase (cf. quinolyl-8-glucuronide; Friedenwald & Becker, 1948; Burton & Pearse, 1952; Robinson, Smith & Williams, 1953*a*).

Chemically, the seven monohydroxyquinolines fall into two groups. The 3-, 5-, 6-, 7- and 8-hydroxyquinolines behave like phenols whereas the 2- and 4-derivatives react mainly as quinolones (see Elderfield, 1952). In this paper, it will be shown that these differences in chemical behaviour are also evident during their metabolism in the rabbit, since the phenolic quinolinols are conjugated directly with glucuronic acid to form quinolylglucuronides, whereas the 2- and 4-quinolones are apparently partly hydroxylated, in an unknown position *x*, to hydroxyquinolones which are then excreted as quinolone glucuronides and ethereal sulphates.

The preparation of quinolyl-8-glucuronide has already been described (Brahm, 1899; Robinson *et al.* 1953*a*). That 3-quinolinol is excreted conjugated in the dog has been shown by Novack & Brodie (1950), but the conjugate was not isolated. Fenyvessy (1900) fed 2-quinolone (carbostyril) to rabbits and isolated a glucuronide which he described as carbostyril glucuronide, but it will be shown that this glucuronide is probably a derivative of a hydroxycarbostyril. The metabolism of the other hydroxyquinolines has not so far been studied. The 3- and 6-quinolinols, however, have received attention as possible therapeutic agents (Davin & Schlauch, 1932; von Jacksch, 1884) and 8-quinolinol (oxine) has been used as an antiseptic and fungicide (Albert, 1951; Frear, 1951). 8-Quinolinol is reported by Root & Chen (1952) to produce pancreatic diabetes occasionally in rabbits, but no ill effects were noted in the present experiments.

Since the chemical differences between the quinolinols and quinolones are analogous to those between 3-hydroxypyridine and the pyridones, it was of interest to study the conjugation of the hydroxypyridines in the rabbit. Anderton, Smith & Williams (1948) observed that only 3-hydroxypyridine formed appreciable amounts of ethereal sulphate in the rabbit and it will be shown that only this isomer is conjugated to any appreciable extent with glucuronic acid.

EXPERIMENTAL

Melting points are uncorrected and optical rotations are given in Table 5. Ultraviolet-absorption spectra were measured in a Unicam S.P. 500 spectrophotometer.

Table 1. *The excretion of glucuronic acid and ethereal sulphate by rabbits after oral doses of pyridine, quinoline and their monohydroxy derivatives*

(Dose 250 mg./kg. Each value gives average result from three rabbits, except those for 5-quinolinol on one animal only.)

	Percentage of dose excreted combined with	
	Glucuronic acid	Ethereal sulphate
Conjugated directly:		
3-Quinolinol	40	5
5-Quinolinol	19	12
6-Quinolinol	54	4
7-Quinolinol	55	6
8-Quinolinol	20	6
Hydroxylated and then conjugated:		
Quinoline	40	8
2-Quinolone	27	23
4-Quinolone	26	6
Pyridine	0	0
2-Pyridone	4	4
3-Pyridol	30	11
4-Pyridone	0·8	0·5

Preparation of compounds. 8-Quinolinol (m.p. 75°) and 2-quinolone (m.p. 200°) were purchased. The 3- and 5-quinolinols, prepared from the corresponding amino compounds by diazotization (Mills & Watson, 1910), had m.p. 197 and 225–230° respectively after crystallization from methanol. 4-Quinolinol, m.p. 200°, was prepared by decarboxylation of kynurenic acid (Albert & Magrath, 1947). The 6- and 7-quinolinols, prepared by Cohen's (1930) modification of the Skraup synthesis, had m.p. 193 and 238° (decomp.) respectively after crystallization from dioxan. 2-Pyridone, m.p. 110°, was purchased. 3-Hydroxypyridine,

m.p. 128°, was prepared by alkaline fusion of the 3-sulphonic acid (Weidel & Murmann, 1895). 4-Pyridone, m.p. 149°, was prepared from chelidamic acid (King & Ware, 1939).

Methods. Compounds were administered orally by stomach tube to rabbits kept as previously described (e.g. Kamil, Smith & Williams, 1951). The glucuronides were isolated from the urine by the methods of Kamil *et al.* (1951) unless they crystallized directly from the urine. Glucuronic acid was estimated by Mills & Paul's modification (cf. Paul, 1951) of the naphthoresorcinol reaction and ethereal sulphates by the method of Sperber (1948). The results are given in Table 1.

Isolation of the glucuronides

2-Quinolonyl-x-glucuronide. 2-Quinolone (2 g.) was fed to two rabbits. The urine was collected for 24 hr. (400 ml.), acidified with 5 ml. of glacial acetic acid and kept at 0° for 2 weeks. The crystalline deposit (1·3 g., 13% of dose) was collected and recrystallized from hot water. The same product was also obtained through the basic lead salt as described by Fenyvessy (1900). *β-2-Quinolonyl-x-D-glucuronide* formed short colourless needles, m.p. 250–252° (decomp.). (Fenyvessy's carbostyril glucuronide 'darkened at 220° and charred at 250–252°'.) Its solubility in water at room temperature was less than 0·1% (w/v) and it was insoluble in ethanol. (Found: C, 50·6; H, 4·8; N, 4·1; H_2O, 5·2. $C_{15}H_{15}O_8N$, H_2O requires C, 50·7; H, 4·8; N, 4·0; H_2O, 5·1%.) The glucuronide (0·2 g.) was treated with 2 ml. conc. HCl and gently warmed. On cooling, colourless needles of the *hydrochloride* separated, m.p. 190–200° (decomp.). (Found: C, 47·7; H, 4·8. $C_{15}H_{15}O_8N.HCl$ requires C, 48·2; H, 4·3%.) This compound was very sparingly soluble in cold water presumably due to hydrolysis. The potassium salt of the glucuronide crystallized as colourless plates from aqueous ethanol and decomposed at 250–260°. (Found: C, 39·5; H, 4·7. $C_{15}H_{14}O_8NK$, $4{\cdot}5H_2O$ requires C, 39·5; H, 5·1%.) A K salt was also described by Fenyvessy (1900).

Quinolyl-3-glucuronide. Two rabbits were each fed 2 g. of 3-quinolinol, and 2 g. of crude glucuronide gum were obtained from the 24-hr. urine through the basic lead salt. This gum did not crystallize but on neutralization with K_2CO_3 in 10 ml. of water and cautious addition of ethanol a precipitate started to form, which on keeping at 0° gave a small crop of crystals mixed with some gum. Recrystallization from aqueous ethanol and then from aqueous dioxan yielded the *potassium salt* of *β-quinolyl-3-D-glucuronide* as clusters of needles very soluble in water and insoluble in ethanol. (Found: C, 47·3; H, 4·5; N, 3·6; K, 10·6; H_2O, 4·3. $C_{15}H_{14}O_7NK$, H_2O requires C, 47·7; H, 4·3; N, 3·7; K, 10·4; H_2O, 4·8%.)

4-Quinolonyl-x-glucuronide. The glucuronide gum (5 g.) was obtained through the basic lead salt from the 24-hr. urine of three rabbits which had collectively received 6 g. of 4-quinolone. This gum did not crystallize directly, but after neutralization with Na_2CO_3 in 10 ml. water and cautious addition of ethanol, a crystalline Na salt separated and was recrystallized from 50% (v/v) aqueous ethanol. (Found: C, 42·7; H, 4·9; N, 3·6; Na, 5·5; H_2O, 14·2. *Sodium salt* of *β-4-quinolonyl-x-D-glucuronide*, $C_{15}H_{14}O_8NNa$, $3{\cdot}5H_2O$ requires C, 42·7; H, 5·0; N, 3·3; Na, 5·4; H_2O, 14·9%.) On drying at 110° it lost water but the anhydrous salt was very hygroscopic and rapidly rehydrated on standing in the air. Yield (three experiments) 0·5–1% of the dose fed. The Na salt (50 mg.) was dissolved in 0·1 ml. of warm water and a drop of glacial acetic acid added. On cooling, free *β-4-quinolonyl-x-D-glucuronide* separated as a mass of long needles, m.p. 208° (decomp.). (Found: C, 46·1; H, 5·5; N, 3·5. $C_{15}H_{15}O_8N$, $3H_2O$ requires C, 46·0; H, 5·4; N, 3·6%.) The ethanolic mother liquor from the above Na salt, when treated with an excess of absolute ethanol, yielded an amorphous precipitate which appeared to be the Na salt of another glucuronide which did not yield 4-quinolone on hydrolysis. The structures of these two glucuronides are being investigated.

Quinolyl-5-glucuronide. 5-Quinolinol (1·5 g.) was fed to a rabbit and the glucuronide isolated through its basic lead salt from the 24-hr. urine as a clear yellow syrup. This was dissolved in hot 50% aqueous ethanol and, on cooling, the partially crystalline, greenish yellow precipitate which formed was filtered off and converted into the K salt which crystallized from 50% ethanol as colourless plates (yield 200 mg. or 6% of the dose). This appeared to be the *potassium salt* of *β-quinolyl-5-D-glucuronide.* (Found: C, 47·2; H, 4·7; N, 3·5; K, 10·8. $C_{15}H_{14}O_7NK$, H_2O requires C, 47·7; H, 4·3; N, 3·7; K, 10·4%.)

Quinolyl-6-glucuronide. The 24-hr. urine (220 ml.) from two rabbits which each received 2 g. of 6-quinolinol was acidified with a few ml. of glacial acetic acid and left at 0° for 24 hr. The crop of colourless crystals was removed and recrystallized from 50% aqueous ethanol containing a trace of acetic acid (yield, 1·7 g. or 17% of the dose). *β-Quinolyl-6-D-glucuronide dihydrate* formed long colourless needles, sparingly soluble in ethanol and water, m.p. 205° (decomp.). (Found: C, 50·6; H, 5·5; N, 3·7. $C_{15}H_{15}O_7N$, $2H_2O$ requires C, 50·4; H, 5·4; N, 3·9%.)

Quinolyl-7-glucuronide. Urine from two rabbits which had each received 2 g. of 7-quinolinol yielded, through the basic lead salt, the glucuronide as a light-brown gel containing some crystals. This was dissolved in 20 ml. of warm 50% aqueous ethanol and on cooling slowly to 0° the solution set to a mass of crystals (yield, 5 g. or 50% of the dose). Recrystallized from water, *β-quinolyl-7-D-glucuronide* formed colourless needles, m.p. 225° (decomp.). (Found: C, 48·9; H, 5·2; N, 4·2; H_2O, 12·2. $C_{15}H_{15}O_7N$, $2{\cdot}5H_2O$ requires C, 49·2; H, 5·5; N, 3·8; H_2O, 12·2%.) The glucuronide (1 g.) when dissolved in 4 ml. of warm conc. HCl and cooled, yielded the dihydrate of *quinolyl-7-glucuronide hydrochloride* as small colourless needles (0·5 g.). (Found: C, 44·8; H, 4·8; N, 3·6. $C_{15}H_{15}O_7N.HCl$, $2{\cdot}5H_2O$ requires C, 44·7; H, 5·3; N, 3·5%.)

Quinolyl-8-glucuronide. Quinolyl-8-glucuronide was prepared according to Robinson *et al.* (1953*a*). The *hydrochloride* was prepared as for the 7-isomer. (Found: C, 45·5; H, 5·0; Cl, 9·5. $C_{15}H_{15}O_7N.HCl$, $2H_2O$ requires C, 45·7; H, 5·1; Cl, 9·0%.)

3-Pyridinol. The 24-hr. urine of a rabbit which had received 1·2 g. of 3-pyridinol yielded through the basic lead salt 1·4 g. of crude glucuronide gum. This was treated with 0·3 g. of K_2CO_3 in 3 ml. water, and ethanol was added until turbid. On keeping at 0°, the solution deposited crystals. The *potassium salt* of *β-pyridyl-3-D-glucuronide monohydrate* recrystallized from aqueous ethanol as colourless plates. (Found: C, 40·4; H, 4·4; N, 3·6; K, 12·3. $C_{11}H_{12}O_7NK$, H_2O requires C, 40·4; H, 4·3; N, 4·3; K, 12·0%.) It showed $\epsilon_{max.}$ 2900 at 270 mμ. in 0·1 N-NaOH and $\epsilon_{max.}$ 4880 at 274 mμ. in 0·1 N-HCl.

Table 2. *Colour reactions and R_F values of hydroxyquinolines*

(Solvent mixture: *A*, *n*-butanol (5 vol.), benzene (2 vol.), ammonia, sp.gr. 0·880 (2 vol.); *B*, *n*-butanol (4 vol.), acetic acid (1 vol.), water (5 vol.). Whatman no. 1 paper. Run until solvent front had moved 12 in. from starting line.)

	R_F value in solvent mixture		Colour with		Fluorescence in ultraviolet light in 0·1 N-HCl or 0·1 N-NaOH
	A	*B*	Brentamine Fast Blue + $NaHCO_3$	$FeCl_3$	
2-Quinolone	0·9	0·9	None	Orange	Purple
3-Quinolinol	0·6	0·9	Red	Red	Blue
4-Quinolone	0·9	0·8	None	Red	Pale purple
5-Quinolinol	0·8	0·8	Red-brown	Green	Pale yellow-green*
6-Quinolinol	0·8	0·8	Red	Brown	Pale yellow
7-Quinolinol	0·7	0·8	Purple	Brown	Bright green
8-Quinolinol	0·9	0·8	Purple	Green	None

* None in 0·1 N-NaOH.

Hydrolysis of the quinolylglucuronides and identification of the quinolinols by paper chromatography

The R_F values of the seven isomeric quinolinols in two solvent mixtures are given in Table 2. The quinolinols were located by their characteristic fluorescence in ultraviolet light, by their colour reaction with $FeCl_3$ and with Brentamine Fast Blue B salt (tetraazotized di-*o*-anisidine). The quinolylglucuronides do not give colours with $FeCl_3$ or Brentamine Fast Blue (Robinson *et al.* 1953*a*) and they fluoresce differently from the free quinolinols (Table 3).

Table 3. *Ultraviolet fluorescence of the quinolylglucuronides*

β-Glucuronide	Colour of fluorescence in 0·1 N-HCl	Colour of fluorescence in 0·1 N-$NaHCO_3$
2-Quinolonyl-*x*-	Purple	Purple
Quinolyl-3-	Purple	Blue
4-Quinolonyl-*x*-	Purple	Purple
Quinolyl-5-	Pale green	None
Quinolyl-6-	Blue	None
Quinolyl-7-	Purple	Blue
Quinolyl-8-	Blue-green	None

A few mg. of each glucuronide were evaporated on a steam bath with 1 ml. of conc. HCl and the residue taken up in a few drops of water and chromatographed on paper. In the case of 3-, 5-, 6-, 7- and 8-quinolylglucuronides the corresponding quinolinols were identified in the hydrolysate. The same results were obtained when the glucuronides were hydrolysed by incubating with locust β-glucuronidase (Robinson *et al.* 1953*a*). The glucuronides isolated after feeding 2- and 4-quinolones, however, gave hydrolysis products differing in R_F and in colour reactions from the material administered. The structure of these glucuronides is being investigated.

DISCUSSION

All the phenolic quinolinols (I; OH at 3, 5, 6, 7 or 8) appear to conjugate directly with glucuronic and sulphuric acids (II; X = $C_6H_9O_6$ or SO_3H). The sum of these conjugations, however, does not account for more than 50–60 % of the dose (Table 1). In all cases the sulphate conjugation is low and phenolic quinolinols appear to be mainly excreted as glucuronides, all of which have been isolated and characterized. The 2- and 4-quinolones (III*a*, *b*; O at 2 or 4) behave differently from their phenolic isomers since they do not conjugate directly but are first hydroxylated elsewhere in the molecule, i.e. in positions 3, 5, 6, 7 or 8 and are excreted as conjugates of hydroxyquinolones (IV; position of OX arbitrary). This suggests that there is insufficient of the hydroxylated or lactim form (III*a*) present to permit conjugation and biologically the quinolones behave as if they were entirely in the keto or lactam form (III*b*). The pyridinols behave similarly (cf. Anderton *et al.* 1948). 3-Pyridinol (V), in agreement with its phenolic character, conjugates with both glucuronic and sulphuric acids (VI), whereas the non-phenolic 2- and 4-pyridones (VII*a*, *b*; O at 2 or 4) hardly conjugate at all. In fact, nothing is

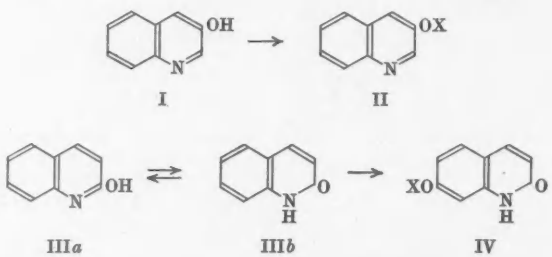
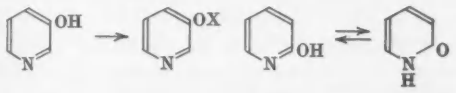


Table 4. *Maximum values from absorption spectra of quinolylglucuronides*

β-Glucuronide	In 0·1 N-HCl						In 0·1 N-NaOH					
	$\lambda_{max.}$ (mμ.)	$\epsilon \times 10^{-3}$	$\lambda_{max.}$ (mμ.)	$\epsilon \times 10^{-3}$	$\lambda_{max.}$ (mμ.)	$\epsilon \times 10^{-3}$	$\lambda_{max.}$ (mμ.)	$\epsilon \times 10^{-3}$	$\lambda_{max.}$ (mμ.)	$\epsilon \times 10^{-3}$	$\lambda_{max.}$ (mμ.)	$\epsilon \times 10^{-3}$
2-Quinolonyl-*x*-	—	—	270	9·2	336	6·9	232	54·7	—	—	340	6·5
Quinolyl-3-	240	26·4	315	5·3	—	—	232	31·7	280	3·0	315	3·5
4-Quinolonyl-*x*-	235	52·4	298	5·0	320	5·5	240	30·4	308	6·8	320	7·0
Quinolyl-5-	245	39·1	315	3·3	345	2·9	235	36·6	—	—	300	4·0
Quinolyl-6-	243	36·4	315	6·5	—	—	220–5	36·2	275	3·3	315	3·3
Quinolyl-7-	240	36·9	~310	4·8	330	7·0	228	39·0	265	2·9	315	4·2
Quinolyl-8-	245	46·6	313	3·8	335	2·5	234	44·1	~262	2·3	300	4·4

~ denotes an inflexion.

known about their metabolism. Pyridine itself does not stimulate the excretion of extra glucuronic acid and ethereal sulphate and if pyridine is oxidized in the rabbit, it may well form one or both pyridones. A pyridone is formed in man during the metabolism of nicotinamide (Knox & Grossman, 1947).



V VI VII*a* VII*b*

Quinoline, unlike pyridine, forms large amounts (40% of the dose) of glucuronide and a small amount (8%) of ethereal sulphate. This suggests that quinoline is at least oxidized in one or more of the positions giving rise to phenolic quinolinols, i.e. 3, 5, 6, 7 and 8 (cf. Novack & Brodie, 1950); it may also be oxidized in the 2- and 4-position, but this type of oxidation would not result in the excretion of extra glucuronic acid. In fact, Knox (1946) has shown that quinoline is oxidized *in vitro* by a rabbit liver preparation to 2-quinolone. The metabolism of quinoline in the intact rabbit is being further investigated.

The assignment of a glucuronosidoquinolone structure (IV) to the metabolites of 2- and 4-quinolones is supported by the nature of their absorption spectra (Table 4). The phenolic glucuronides show specific light absorption in the 230–240 and 290–330 mμ. regions and the spectra show the bathochromic shifts characteristic of quinolyl ethers in general on moving from alkaline or neutral to acid solution (cf. Ewing & Steck, 1946). The glucuronides from the quinolones, on the other hand, do not show this shift and the wavelength of maximum absorption is little different in acid and alkali (Table 4). According to Ewing & Steck (1946) this is characteristic of the intact quinolone structure and suggests that the glucuronic acid is attached through the newly introduced hydroxyl rather than through the oxygen atom at the 2- or 4-position. The glucuronide from 4-quinolone also exhibits the divided maximum at 300–320 mμ. noted by Ewing & Steck in several substituted 4-quinolones but which is not present in the 4-quinolyl ethers (Fig. 1). The quinolone structure is also supported by the values found for the basic ionization constants of the glucuronides from 2- and 4-quinolone. While the quinolyl glucuronides have basic pK_a values of

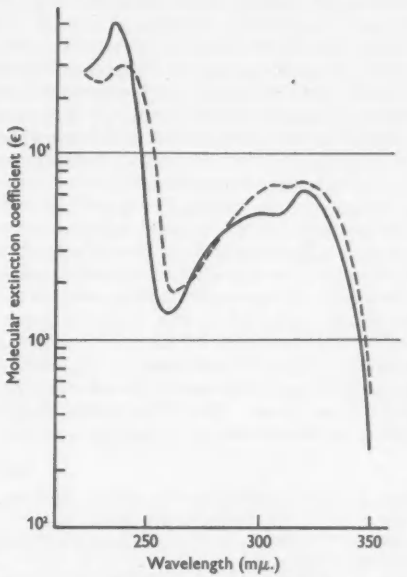


Fig. 1. Absorption spectra of 4-quinolonyl-*x*-glucuronide in 0·1 N-HCl (—)and 0·1 N-NaOH (– – –).

about 5 (Robinson, Smith & Williams, 1953*b*), near those of quinoline and quinolyl ethers (Veley, 1904; Keneford, Morley, Simpson & Wright, 1949), the glucuronosidoquinolones are weaker bases and have pK_a values of about 3·7, near to the value of 2·4 found by Keneford *et al.* for 4-quinolone, and 4·3 reported by Veley for 2-quinolone.

The main purpose of this work, however, was the preparation of the quinolyl glucuronides as reference compounds for work on the metabolism of quinoline.

Table 5. *The optical rotations of the quinolylglucuronides*

(Observations were made on 1% solutions unless the concentration is given in parentheses. The error in $[\alpha]_D$ when $c=1$ is $\pm 3°$.)

	In water		In 0·1 N-NaOH	
β-Glucuronide	$[\alpha]_D^{20}$ (°)	$[M]_D^{20}/100$ (°)	$[\alpha]_D^{20}$ (°)	$[M]_D^{20}/100$ (°)
2-Quinolonyl-*x*-	−99 (*c*, 0·05)	—	−97	−344
K salt of 2-quinolonyl-*x*-	−75 (*c*, 2·5)	—	—	—
Hydrochloride of 2-quinolonyl-*x*-	−66 (*c*, 0·1)	—	—	—
K salt of quinolyl-3-	−94	−354	−92	−348
4-Quinolonyl-*x*-	−128 (*c*, 0·015)	—	—	—
Na salt of 4-quinolonyl-*x*-	−111	−468	—	—
K salt of quinolyl-5-	−71	−268	−73	−276
Quinolyl-6-	−114 (*c*, 0·1)	—	−108	−348
Quinolyl-7-	−66·5	−243	−97	−346
Hydrochloride of quinolyl-7-	−90	−353	—	—
Quinolyl-8-	−75 (*c*, 0·1)	—	−88	−350
Hydrochloride of quinolyl-8-	−77·2 (*c*, 0·2)	—	—	—
K salt of pyridyl-3-	−81	−265	—	—

These compounds are easy to identify since they have a marked fluorescence in ultraviolet light which in many cases changes characteristically on moving from acid to alkaline solution (Table 3). This property may also be of use in the enzymic study of glucuronide synthesis since quinolyl-8-glucuronide can be detected readily by its fluorescence in the presence of excess 8-quinolinol which does not itself fluoresce. The easily prepared 6- and 7-quinolylglucuronides may also serve as alternative substrates to quinolyl-8-glucuronide in glucuronidase estimations (cf. Robinson *et al.* 1953*a*). The quinolinols, when liberated from the conjugates, all give intense colours with Brentamine Fast Blue (Table 2). The glucuronides do not give a colour with this reagent.

The specific and molecular optical rotations of the glucuronides prepared in this work are given in Table 5. The molecular rotations of the glucuronides (except that of 5-quinolinol) in 1% solution in 0·1 N-sodium hydroxide are of the same order, i.e. $[M]_D^{20}/100$ is about $-350°$. This means that the position of attachment of glucuronic acid to the quinoline nucleus (other than positions 2 and 4) has little influence on the molecular rotation.

SUMMARY

1. Measurements have been made of the excretion of glucuronic acid and ethereal sulphate by rabbits dosed with pyridine, quinoline, and each of the monohydroxy derivatives.

2. 3-Hydroxypyridine and 3-, 5-, 6-, 7- and 8-hydroxyquinolines conjugate directly to form glucuronides which have been isolated and characterized.

3. 2- and 4-Quinolones are hydroxylated by the rabbit and excreted as quinolonylglucuronides which have been isolated.

4. The ultraviolet-absorption spectra of the glucuronides and their characteristic fluorescence in ultraviolet light have been recorded.

The author wishes to thank Prof. R. T. Williams for his encouragement and interest in this work, Dr D. Robinson for the measurement of some of the spectra quoted, and Dr G. T. Mills and Dr J. Paul for details of their modification of the naphthoresorcinol method.

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The Effect of pH on the Affinities of Enzymes for Substrates and Inhibitors

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There are five types of equilibria, representing reversible combinations, which are especially important in biochemistry. They are as follows (see Table 1): (*a*) acid-base equilibria: combination of acid anions with hydrogen ions; (*b*) oxidation-reduction equilibria: combination of oxidants with hydrogen; (*c*) phosphorylation equilibria: combination of organic molecules with inorganic phosphate; (*d*) Michaelis complex formation: combination of enzymes with their substrates; (*e*) inhibition equilibria: combination of enzymes with reversible inhibitors.

All these equilibria are formally similar; they obey equations which are identical in form, and are amenable to the same treatment. It has been shown (Dixon, 1949) in the case of the hydrogenation and phosphorylation equilibria that when the systems are plotted on a logarithmic scale with respect to the second reactant shown in column 2 of Table 1 (H_2 or P respectively), a number of simple and useful rules can be formulated which greatly facilitate the interpretation of the experimental data. It is the purpose of the present paper to show that if the systems of the last two types are similarly represented, rules of identical form can be applied to them. It appears probable that these rules may throw a good deal of light on the mechanism of enzyme-substrate and enzyme-inhibitor combination and on the nature of the chemical groups involved, and that a profitable new field for enzyme work may be opened up.

THEORY

Scale definitions

For the treatment of the five types of equilibria (Table 1), five different but analogous scales are already in use, represented by the symbols pH, rH, rP, pS and pI respectively. In each case the scale value is defined as $-\log_{10} c$, where c is the concentration (or more strictly the activity) of the 'scale-defining substance', i.e. the second reactant shown in column 2. For hydrogen the activity is expressed in atmospheres, for the other cases, in molarities. The scale definitions are shown in column 3 of the table.

No new symbols are required and these symbols are used with their accepted meanings. It will be noticed as we proceed that when the various sets of data are brought together in such a unified scheme the symbols do not form as consistent a system as one could wish. The reason is that they have been introduced at different times by a number of workers, all concerned with different systems.

There is a slight theoretical difference between the last two types and the others. For each of the first three types the scale-defining substance is the same for all the systems in that group. All acid anions are in equilibrium with hydrogen ions, all the hydrogenated systems with hydrogen and all the phosphorylated systems with phosphate. All enzymes, however, are not in equilibrium with the same substrate; on the contrary, we are concerned with a different substrate for each enzyme. But this point is of no importance in practice, for we are almost invariably concerned with only one enzyme at a time in this connexion, and have not to consider the distribution of a given substrate between a number of enzymes.

Effect of relative concentrations of components

Putting the equilibrium equations into their logarithmic forms we obtain the following five equations

$$\left.\begin{aligned} \mathrm{pH} &= \mathrm{p}K - \log\frac{[A\mathrm{cH}]}{[Ac^-]},\\ \mathrm{rH} &= \mathrm{rH}_0 - \log\frac{[A\mathrm{H}_2]}{[A]},\\ \mathrm{r}P &= \mathrm{r}P_0 - \log\frac{[XP]}{[X]},\\ \mathrm{p}S &= \mathrm{p}K_{s0} - \log\frac{[ES]}{[E]},\\ \mathrm{p}I &= \mathrm{p}K_{i0} - \log\frac{[EI]}{[E]}. \end{aligned}\right\} \qquad (1)$$

If therefore we plot the first of these quantities in each case against the composition of the systems, i.e. the degree of reduction, phosphorylation, etc., we obtain in every case the same curve of the familiar form shown in Fig. 1. This curve represents (*a*) the degree of association of the acid in terms of the pH, (*b*) the degree of reduction (or hydrogenation) of the

Table 1. *Formulations and symbols for various equilibria*

Column 1	2	3	4	5
Type	Equilibrium	Scale definitions	Mid-point without ionization	Mid-point with ionization
(*a*) Acid-base	$Ac^- + H^+ \rightleftharpoons AcH$	$pH = -\log [H^+]$	pK	—
(*b*) Oxidation-reduction	$A + H_2 \rightleftharpoons AH_2$	$rH = -\log [H_2]$	rH_0	$r'H$
(*c*) Phosphorylation	$X + P \rightleftharpoons XP$	$rP = -\log [P]$	rP_0	$r'P$
(*d*) Enzyme-substrate	$E + S \rightleftharpoons ES$	$pS = -\log [S]$	pK_{s0}	pK_s
(*e*) Enzyme-inhibitor	$E + I \rightleftharpoons EI$	$pI = -\log [I]$	pK_{i0}	pK_i

oxidation-reduction system in terms of the rH, (*c*) the degree of phosphorylation of an organic system in terms of the rP, (*d*) the degree of 'substration' of an enzyme (if we may coin a word) in terms of the pS, and (*e*) the degree of inhibition of an enzyme in terms of the pI.

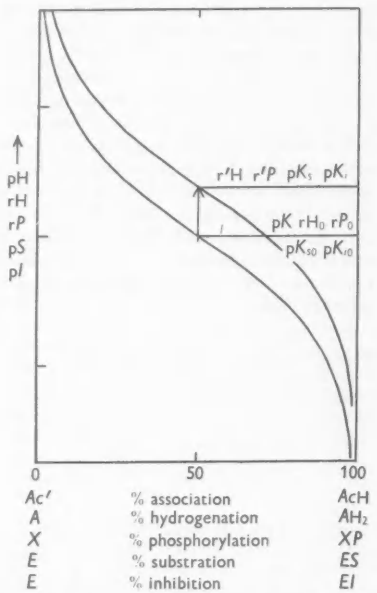

Fig. 1. Effect of alteration of composition of the five types of system (Table 1). The arrow represents the effect of ionization.

When there are no complications due to the effects of ionization of the components, which will be dealt with below, the mid-points of the curves are given by the middle terms of equations (1), as shown in column 4 of the table. The symbols have their usual meanings, and pK_{s0} and pK_{i0} denote minus the logarithm of the Michaelis constant and inhibitor constant respectively, the subscripts '$_0$' showing that the quantities refer to the non-ionized systems. The term 'Michaelis constant' is used in this paper in its original sense as defined by Michaelis & Menten (1913), i.e. as the dissociation constant of the enzyme-substrate complex, or in other words the reciprocal of the enzyme-substrate affinity. To avoid any confusion the earlier symbol K_s for this equilibrium constant will be used here, rather than the commonly used K_m, as in recent years the latter has come to be used to denote the substrate concentration giving half the maximum velocity, a less fundamental but more easily measured quantity which may be partly kinetic in nature. K_s and K_m are equal, except in those cases which require the use of the Briggs-Haldane (1925) theory, where the kinetic element in K_m causes it to diverge from the true equilibrium constant K_s.

Free energy and heat of formation of ES *and* EI *complexes*

The same rules as for rH and rP apply (see Dixon, 1949, pp. 70, 72, 93–4; rules 3 and 8). Thus the standard free energy of formation of the Michaelis complex is given by $-\Delta G^\circ = 2{\cdot}3\boldsymbol{R}T.pK_{s0}$ (or, if there is ionization, by $2{\cdot}3\boldsymbol{R}T.pK_s$ (see next section) the value then including the free energy of the ionization reactions).

The heat of combination of enzyme with substrate is given by $\Delta H = 2{\cdot}3\boldsymbol{R}T^2 \frac{d(pK_s)}{dT}$.

Similar rules, with substitution of K_i for K_s, apply to the formation of inhibitor-enzyme complexes.

Effect of ionization of the components

In the case of rH and rP it has been shown that ionization of one or more of the components of the system causes a displacement of the curve to an extent depending on the pH, so that the mid-point is shifted along the scale from the position (rH_0 or rP_0) which it had in the absence of ionization effects, to new positions denoted by r'H and r'P respectively, as shown by the arrow in Fig. 1. These effects are revealed when we plot the values of the half-hydrogenated or the half-phosphorylated system (i.e. r'H or r'P) against the pH. In what follows we are no longer concerned with the first line of the table, since it is meaningless to plot pH against pH.

The deviations caused by ionization are due to the fact that when there is ionization the amount of the unionized form of a given component (to which equations (1) refer) is no longer equal to the total amount of the component taken, because the total concentration is the sum of the concentrations of the unionized form and all the ionized forms. It is therefore necessary to obtain an expression for the concentration of the unionized form of the substance in terms of its total concentration, its ionic dissociation constants and the pH, and to substitute this in equations (1).

Taking an rH system as an example, the molecule of the reduced component AH_2 (which might represent some such substance as quinol) might ionize as a dibasic acid with dissociation constants K_1 and K_2. Its total concentration would then be given by $Re_t=[AH_2]+[AH^-]+[A^{2-}]$ and therefore $Re_t=[AH_2].f(\text{pH})$, where $f(\text{pH})$ is a function of the pH and the dissociation constants of AH_2. Substituting in the second of equations (1) we get

$$\text{rH}=\text{rH}_0-\log\frac{Re_t}{Ox_t}+\log f(\text{pH}).$$

For the half-reduced system $Re_t=Ox_t$, so that $\text{r}'\text{H}=\text{rH}_0+\log f(\text{pH})$ and the last term represents the amount of the deviation of the mid-point from rH_0 due to the ionization. Following Michaelis (1930) we shall use the subscript t to denote the total concentration of all ionization states of a given substance.

In the case of a dibasic acid like quinol, $\log f(\text{pH})$ is equal to $\log\left(1+\frac{K_1}{[\text{H}^+]}+\frac{K_1K_2}{[\text{H}^+]^2}\right)$ (see Dixon, 1949, p. 74).

The treatment of the enzyme systems is exactly analogous. The substrate, the enzyme and the enzyme-substrate complex may all ionize. In order to illustrate the treatment let us assume, for the sake of simplicity, that each has two acidic groups like quinol. Then if E_t denotes the total concentration of free enzyme (not the total enzyme concentration) and S_t the total free substrate concentration,

$$\left.\begin{aligned}
E_t&=[E]+[E^-]+[E^{2-}]=[E]\left(1+\frac{K_{e1}}{[\text{H}^+]}+\frac{K_{e1}K_{e2}}{[\text{H}^+]^2}\right)=[E].f_e(\text{pH}),\\
S_t&=[S]+[S^-]+[S^{2-}]=[S]\left(1+\frac{K_{s1}}{[\text{H}^+]}+\frac{K_{s1}K_{s2}}{[\text{H}^+]^2}\right)=[S].f_s(\text{pH}),\\
ES_t&=[ES]+[ES^-]+[ES^{2-}]=[ES]\left(1+\frac{K_{es1}}{[\text{H}^+]}+\frac{K_{es1}K_{es2}}{[\text{H}^+]^2}\right)=[ES].f_{es}(\text{pH}).
\end{aligned}\right\}\qquad(2)$$

K_{s1}, K_{s2} (the acidic dissociation constants of the substrate) should not be confused with K_s.

The ordinary Michaelis constant K_s is the equilibrium constant of the enzyme-substrate complex formation, expressed in terms of the total concentrations of the components, whereas the constant K_{s0} which appears in the fourth of equations (1) is the corresponding constant for the unionized forms, therefore

$$K_s=\frac{E_t.S_t}{ES_t}=\frac{[E]\,[S]}{[ES]}\frac{f_e(\text{pH}).f_s(\text{pH})}{f_{es}(\text{pH})}=K_{s0}\frac{f_e(\text{pH}).f_s(\text{pH})}{f_{es}(\text{pH})},$$

or in logarithmic form

$$\text{p}K_s=\text{p}K_{s0}+\log f_{es}(\text{pH})-\log f_e(\text{pH})-\log f_s(\text{pH}).\qquad(3)$$

The functions $f(\text{pH})$ are identical with those appearing in the corresponding rH and rP equations, having the form 1, $1+\frac{K}{[\text{H}^+]}$, $1+\frac{K_1}{[\text{H}^+]}+\frac{K_1K_2}{[\text{H}^+]^2}$, etc., according to whether the substance has 0, 1, 2, etc., ionizing groups. With the appropriate substitution of symbols, equation (3) is identical with that previously given for the other type of system (cf. Dixon, 1949, p. 95).

The treatment does not imply any assumption about the actual mechanism of combination, or which particular ionic form of the substrate it is which combines with the enzyme. It is simply assumed that there is a relation between the concentrations of the unionized components such that $\frac{[E]\,[S]}{[ES]}=\text{constant}$. But since all the ions are in equilibrium with the unionized forms, this implies that there are also similar equilibrium relationships between the corresponding ionic forms. It is easy to show by calculation that the same result is obtained whatever mechanism of combination is assumed.

When there is ionization, the mid-points of all the curves are given by the quantities given in column 5 of Table 1. pK_s, being the point at which the enzyme is 'half-substrated' (or at which $E_t=ES_t$), corresponds to r'H and r'P, the points at which the other systems are half-hydrogenated and half-phosphorylated respectively, and, on plotting pK_s against pH, graphs are obtained which obey the same rules as the r'H and r'P charts previously given, because the functions $f(\text{pH})$ are the same for all systems which ionize similarly (Dixon, 1949, pp. 73 and 98).

These graphs consist of sections of straight lines joined together, each straight part covering a range

of pH in which one of the terms in the functions $f(\mathrm{pH})$ is large compared with the others. Each section will have a zero, 1-unit or 2-unit slope in agreement with the power of $[H^+]$ occurring in the term which is dominant at that pH, and the slope will be positive or negative according to the sign preceding the function in which the term occurs in equation (3). Intermediate values of slope are of course met with in the short curved part at the junction of two sections, where two of the terms are important at the same time. If more than one component of the system ionizes, so that the equation contains two or three pH functions, as in equation (3), the slope will be the algebraic sum of the contributions of all the functions. The term '+1-unit slope' means that the pK_s increases by 1 unit for every increase of 1 unit in the pH.

A very simple example may make this clearer. Let us suppose that the enzyme and the E–S complex do not ionize, but that the substrate has one ionizing group with a pK of 7. On the acid side, well below the pK value, the substrate will be present almost entirely as the unionized form. On the alkaline side, well above the pK, it will be present almost entirely as the anion S^-. Near the pK the substrate will be present as a mixture of the two forms S and S^- in varying proportions.

The functions $f_e(\mathrm{pH})$ and $f_{es}(\mathrm{pH})$ will both be equal to 1 and their logarithms will be zero; $f_s(\mathrm{pH})$ will be $1+K/[H^+]$. Equation (3) therefore takes the form

$$pK_s = pK_{s0} - \log\left(1+\frac{10^{-7}}{[H^+]}\right).$$

Below pH 5, $10^{-7}/[H^+]$ is negligible compared with 1; pK_s is therefore equal to pK_{s0} and independent of the pH in the region where the change on combination is from $E+S$ to ES. Above pH 9, $10^{-7}/[H^+]$ is large compared with 1 and the equation will become $pK_s = pK_{s0} - (\mathrm{pH}-7)$. This represents a straight line with a -1-unit slope intersecting the pK_{s0} line at pH 7, the pK value. In this -1-slope region the change on combination is from $E+S^-$ to ES. In the neighbourhood of the pK neither of the terms within the bracket can be neglected and there is a curved region of the graph. At pH 7, $10^{-7}/H^+$ equals 1, and the curve is therefore log 2 or 0·3 unit below the point of intersection. The graph is of the form shown in Fig. 2*A*.

The rules which permit the easy interpretation of these pH graphs are two in number, the 'rules 9 and 10' of Dixon. The first states that the slope in any pH region is numerically equal to the change of charge on dehydrogenation, dephosphorylation or 'desubstration'; that is to say it is equal to the number of charges on the left-hand side of the equations in column 2 of Table 1 minus the number of charges on the right-hand side, due regard being paid to algebraical sign. For example, in the case of rH, quinol is present in alkaline solutions as the anion Q^{2-}, and the oxidation may be represented as $Q^{2-} \rightarrow Q$, a change of +2 (from two negative charges to zero), and the system gives a +2-unit slope on the rH chart in this pH range. At pH 8 the dephosphorylation of ATP takes place as follows: $ATP^{4-} \rightarrow ADP^{3-} + P^{2-}$, a change of -1, giving a -1-unit slope on the rP chart.

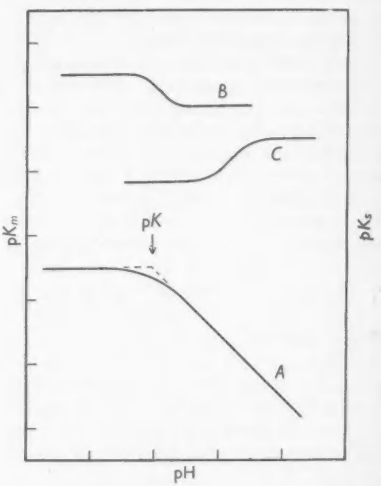

Fig. 2. Diagram to illustrate various effects of pH (see text).

Correspondingly in the case of enzyme systems, if there is no alteration of total charge when the substrate combines with the enzyme, pK_s will not change with pH and the graph will be a horizontal straight line. This would be expected for instance with a non-ionizing substrate, e.g. a carbohydrate, combining with a non-ionized group in the enzyme. It would also be expected where the combination is electrostatic in nature, e.g.

$$R.COO^- + H_3N^+.E \rightarrow R.COO^-.H_3N^+.E.$$

On the other hand, if an acidic substrate were to combine through its acidic group with the enzyme to give a non-ionized compound

$$(R.COO^- + E \rightarrow R.COO.E)$$

there will be a charge change of -1 on 'desubstration' and a -1-unit slope will be expected in the pH range in which the substrate is present as the anion. On the acid side of the pK value of the substrate, however, the substrate will be unionized and the graph will become horizontal as shown in Fig. 2*A*. pK relates to the acid-base ionization of the components and should not be confused with

pK_s or pK_i. Curves of this form have recently been obtained with inhibitors in two cases. Massey (1953) finds that maleate acting on fumarase gives such a curve, with the bend corresponding to the pK of maleate. Chance (1952) gives similar curves for the inhibitors hydrazoic acid and hydrocyanic acid acting on catalase in which the bends agree approximately though not exactly with the respective pK values; in this case the enzyme-inhibitor combination was measured directly by a spectrophotometric method.

This brings us to the second rule, 'rule 10', which states that every change of direction of the graph corresponds to the ionization of a group in one of the components; when the upper side of the bend is the concave side, it indicates a pK (acidic or basic) of the enzyme-substrate complex, while when the concave side faces downwards it indicates a pK of either the free substrate or the free enzyme. This rule can be shown to follow from the fact that in equation (3) the functions $f(\mathrm{pH})$ appear with a positive sign when they refer to substances on the right-hand side of the equilibrium equations in column 2 of Table 1, and with a negative sign when they refer to substances on the left-hand side. Each ionizing group produces a change of one unit in the slope when the pH passes through its pK value and the value of the pK is given by the point of intersection of the straight parts produced, as shown in Fig. 2*A*. In this example, as already mentioned, the ionizing group is present in the free substrate, and it will be noted that the concave side of the bend faces downwards in agreement with the rule.

The curvature at the junction is such that the graph passes through a point 0·3 pK_s unit above or below the point of intersection of the straight parts (Dixon, 1949, p. 76) as shown in Fig. 7. In some cases, however, for reasons which are as yet unexplained, the experimental curves seem to give sharper bends than the theoretical (cf. Fig. 4 and Massey, 1953).

It may seem at first sight that the interpretation of such graphs is subject to ambiguities, since there is nothing to show whether a downward bend represents a pK of the free substrate or of the free enzyme. There is little trouble in practice, however, because in the great majority of cases the pK values of the substrate are known, and downward bends occurring at any other pH values must represent ionizations of the free enzyme.

What is meant by ionization of the enzyme may not be immediately obvious, since, as a protein, it will naturally contain many ionizing groups. It has been pointed out already in the case of the rH systems that groups which ionize equally strongly in the reduced and oxidized forms, e.g. sulphonate groups in dyes, have no effect on the curves. They are not detected and can be disregarded. This is evident from the rule just stated, since groups with the same pK values in the oxidized and reduced forms will produce equal and opposite bends in the curve which will cancel one another out. The same is true in the case of enzymes; a group in the enzyme protein which is equally strongly ionized in the free enzyme and the enzyme-substrate complex will not be shown by the curve. It is only groups whose degree of ionization alters when the enzyme combines with substrate, i.e. groups which form part of the active centre or are sufficiently closely associated with it to be affected when the substrate combines, which are revealed by the curves. This fortunate circumstance enables us to obtain, from the plots of pK_s against pH, information about the active centres, uncomplicated by the ordinary unspecific ionizations which all proteins undergo.

Thus it is essentially the substrate-binding groups in the enzyme which are revealed by the method. It does not follow, however, that all such groups will be shown up. Any substrate-binding group which does not ionize at any accessible pH, either in the free enzyme or the complex, will produce no discontinuities in the curve from which suggestions as to its nature can be obtained.

Various effects may be produced by ionizing groups. If the substrate does not actually combine with the group in question, but merely affects its ionization so that its pK is not the same in the enzyme-substrate complex as it is in the free enzyme, a wave will appear in the curve, as shown in Fig. 2, curve *B*, corresponding to an increase of pK by the substrate and curve *C* to a decrease. It is easily shown that the overall difference in level is then numerically equal to the change in pK. The substrate may combine with an ionized group in the enzyme, forming a non-ionized link, in which case there will be a single downward bend at the pK of the group, giving a curve similar to *A*. On the other hand, a group which is not ionized in the normal substrate may ionize when the substrate becomes activated by combination with the enzyme, giving an upward bend.

The curves may be expected to yield valuable information of three kinds. (*a*) Information about the nature of the enzyme-substrate link, deduced mainly from the slope of the curve: for instance it has been suggested that alkaline phosphatase combines with its substrate by an electrostatic attraction between the negatively charged ionized phosphate group of the substrate and the positively charged Mg atom, somewhat as follows:

$$R.P^{2-} + \mathrm{Mg}^{+}.E \rightarrow R.P^{2-}.\mathrm{Mg}^{+}.E.$$

Since there is no alteration of the total charge, a zero slope would be expected. On the other hand, if one of the ionized OH groups of the substrate combines with the enzyme with the formation of a

non-ionized link, $R.P^{2-} + E \rightarrow R.P^{-}.E$, a -1-unit slope would be expected, and this is what is found in the pH range in which the enzyme is most active.

(*b*) Information about the nature of the substrate-binding groups of the enzyme, deduced from their pK values as determined from the position of the discontinuities of the curve: due caution is of course necessary here; the fact that a substrate-binding group in the enzyme has a pK which is identical with the known pK of some particular group which occurs in proteins is not of itself proof of its nature, because other groups may possibly be found which have the same value of pK. Nevertheless, valuable suggestions may be obtained in this way. Phosphatase is again a case in point, as shown below.

(*c*) Information about the nature of the activation process, deduced from the ionizations of the enzyme-substrate complex: it is not impossible that in certain cases when the substrate becomes activated by the enzyme, certain groups in its molecule may become ionized which normally are non-ionizing. For instance, according to Quastel's (1926, p. 177) theory of the activation of succinic acid by its dehydrogenase, the methylene groups of the activated molecule ionize thus

$$—CH_2— \rightleftharpoons —CH^{-}— + H^{+},$$

and this would be shown by discontinuities in the pK_s curve referable to the enzyme-substrate complex. It is not known whether this is the case.

In order to avoid any misunderstanding it should be pointed out again that the data do not show which of the various forms of the substrate it is which actually combines with the enzyme. The treatment is based on thermodynamic equilibria and does not give the actual mechanism of combination. It gives the overall change of charge when the combination takes place, and the result is precisely the same if the enzyme combines in the first place with, say, the substrate ion as if the unionized substrate first combines and then ionizes. The slope is determined by the forms of the components which are actually present at any pH.

It will be clear that what has been said about enzyme-substrate combination in relation to the pK_s curves applies equally to the reversible combination of enzymes with inhibitors in relation to pK_i curves. The method should yield the same kind of information about inhibitors as about substrates. Indeed it is possible that the study of inhibitors in this way may yield more information about enzymes than the use of substrates, since with any given enzyme the number of inhibitors available for study is usually much larger than the number of substrates. Moreover, it should be possible to obtain information about the groups in the enzyme which combine with particular parts of the substrate molecule by the use of inhibitors containing these parts. This appears to offer a wide field for research.

A comparison of pK_s and pK_i curves for the same enzyme should further assist interpretation, since (unless the presence of the substrate or inhibitor changes the state of the enzyme in some way) the free enzyme will have the same charge in all cases and its pK values should be the same in all the curves.

Measurement of K_s *and* K_i

The preceding sections have been concerned with the theory of the effect of pH on the affinities of enzymes for substrates and inhibitors; they have therefore been concerned only with the equilibria between the components. The practical measurement of these affinities is an entirely separate question, and cannot be dealt with fully within the scope of this paper. Nevertheless, a brief summary of the position may not be out of place here.

Direct measurements of enzyme affinities are possible in certain cases, e.g. where there is a change in absorption spectrum when combination with the enzyme takes place. With enzymes which can be obtained in the pure state in reasonably large amounts it should also be possible to measure the combination by direct chemical estimations in certain cases.

The most widely used methods, however, depend upon measurements of the velocity of the enzyme-catalysed reaction, and the kinetic element in these measurements must not be lost sight of. This kinetic element may in some cases give rise to difficulties in the determination of K_s, though not of K_i. Difficulties in the measurement of K_s do not affect the validity of the theory in any way, though they may sometimes affect its applicability.

The method most generally used for studying affinities for substrates is known as the Lineweaver & Burk graph (1934), which gives K_m, the substrate concentration at which the velocity is half the maximal velocity obtained with high substrate concentration. Whether this is equal to K_s, as would follow from the Michaelis treatment, or whether it diverges from it because of kinetic effects, depends on the relative magnitude of the velocity constants of the system. Writing the reaction as

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + \text{products},$$

it will be clear that if k_3 is small compared with k_1 and k_2, ES will at all times be in equilibrium with E and S. This was the basic assumption of the Michaelis theory, and under these conditions K_m is equal to K_s (i.e. k_2/k_1). In such cases the theory given in this paper, which relates to K_s, can be applied directly to the values of K_m determined by

the Lineweaver method. There is no difficulty in such cases.

In those cases where k_3 is not relatively small, Briggs & Haldane (1925) showed that the observed K_m is equal to $(k_2+k_3)/k_1$ and therefore differs from K_s. It must be emphasized, therefore, that the direct application of the rules given above to the observed K_m values may lead to errors in those cases in which it is necessary to invoke the Briggs-Haldane theory. It may well be, however, that the errors will be confined to a small region in the neighbourhood of the optimum pH where k_3 is large.

Where it is possible to vary k_3 experimentally, e.g. by varying the concentration of a second reactant, K_s may obviously be obtained by extrapolating K_m to $V=0$, V being the maximal velocity obtained in high substrate concentration, which is proportional to k_3. Slater & Bonner (1952) have made use of this principle to determine K_s for succinic dehydrogenase.

Even when the absolute value of K_s cannot be determined it is still possible to obtain some information from the rules by considering together the variation with pH of pK_m and pV (i.e. $-\log V$). It is hoped to deal with the application of the theory to Briggs-Haldane cases more fully in a later paper, but three points may be mentioned here. (1) Rule 10 is still applicable, since the discontinuities in the pK_s graph will also appear in the pK_m graph. (2) In pH regions where the pV curve is horizontal, the participation of k_3 makes no difference to the application of rules 9 and 10, because even though K_m is not equal to K_s, calculation shows that the pK_m graph is parallel to the pK_s graph and is merely displaced from it in the vertical direction. Thus within such regions the slopes are the same and the rules can be applied to the Briggs-Haldane case no less than to the Michaelis case. (3) The occurrence in the pK_m graph of straight portions having whole-number slopes (e.g. zero or 1-unit slopes), in pH regions where the pV graph has non-whole-number slopes or is curved, indicates that k_3 is negligible and the theory can be directly applied to the pK_m graphs in such regions. This is a useful criterion of applicability.

This extension of the theory is still being developed. The grounds for these statements will be given later: they are included here in order to give some idea of the range of applicability of the rules.

No such difficulties arise in the determination of K_i, since the Michaelis equation on which it is based is valid both for the Briggs-Haldane and the Michaelis cases. The reversible inhibitor comes quickly into equilibrium with the enzyme, since the *EI* complex is not used up and there is nothing corresponding to k_3 for the inhibitor. K_i, the actual dissociation constant of the enzyme-inhibitor complex, is conveniently determined by the graphical method of Dixon (1953).

EXAMPLES

A search of the literature shows that experimental work on the variation of Michaelis constants with pH is extremely scanty; in fact the field seems to be almost untouched. The Michaelis constants of a great many enzymes have been determined, but nearly always at one pH value only. In a few cases measurements have been done at two or more pH values, but they are too few, or cover too restricted a pH range, to allow curves to be drawn. The writer has only been able to find seven cases in which the effect of pH on K_m has been studied, namely urease, phosphatase, arginase, saccharase, fumarase, cholinesterase and xanthine oxidase.

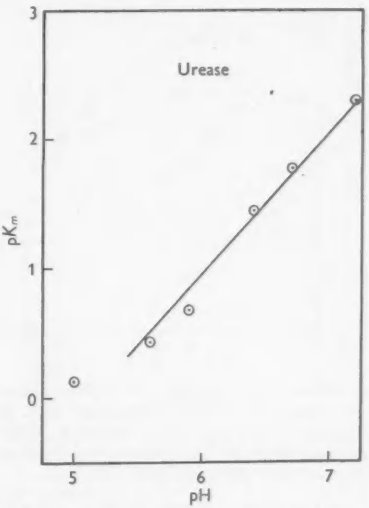

Fig. 3. Effect of pH on pK_m of urease. Plotted from data of Howell & Sumner (1934).

Urease. Howell & Sumner (1934) give a series of curves showing the relation between urea concentration and velocity at different pH values in 0·0125 M-phosphate. The Michaelis constants read from these curves have been plotted as pK_m in Fig. 3, and it will be seen that they fall on a straight line with a +1-unit slope. This would mean either that the enzyme loses one positive (or gains one negative) charge when it combines with urea, or that when the urea is activated by combination with the enzyme it acquires a negative charge. In interpreting these results, however, caution is necessary, because they are only obtained in the presence of

phosphate. In the absence of phosphate the affinities are very much higher and cannot be determined from Howell & Sumner's curves; under these conditions therefore the effect of pH is unknown.

Phosphatase. The lower curve of Fig. 4 is plotted from the data of Jacobsen (1933) on the alkaline phosphatase of kidney, with glycerolphosphate as substrate. A -1-unit slope is obtained, as already mentioned. Recently Morton (1952) has studied highly purified intestinal phosphatase with phenylphosphate as substrate, obtaining the upper curve in Fig. 4. This shows a -1-unit slope on the alkaline

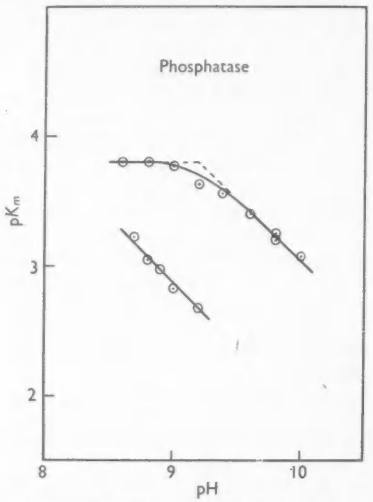

Fig. 4. Effect of pH on pK_m of alkaline phosphatase. Lower curve for kidney phosphatase with glycerolphosphate, plotted from data of Jacobsen (1933); upper curve for highly purified intestinal phosphatase with phenylphosphate, from Morton (1952).

side, but has a discontinuity at about pH 9·2 which is not seen in the other curve. This cannot represent the pK of the substrate, which lies at 5·73 (Delory & King, 1943), and must therefore be due to an ionizing group in the enzyme. The pK value would correspond with a phenolic tyrosine group, an ϵ-amino group, or an SH group; there is independent evidence, however, that SH groups are not involved. It is not certain whether the difference between the two curves is due to the use of different enzymes or of different substrates. The latter seems more probable, for there are indications of a similar discontinuity in the results of Folley & Kay (1935) who used milk phosphatase with phenylphosphate. If so, it suggests that the group in question is concerned in the combination of the enzyme with phenylphosphate but not with glycerolphosphate.

Arginase. The curve shown in Fig. 5 has been plotted from the results of Mohamad & Greenberg (1945) and includes results obtained with ox and

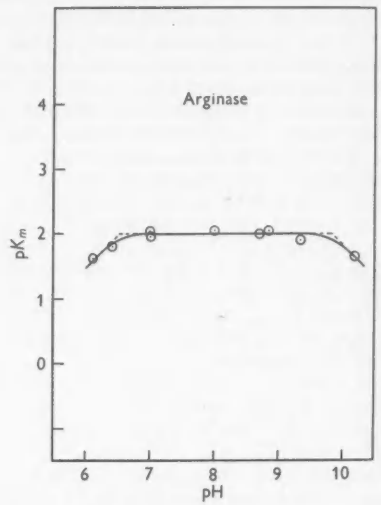

Fig. 5. Effect of pH on pK_m of arginase. Plotted from data of Mohamad & Greenberg (1945).

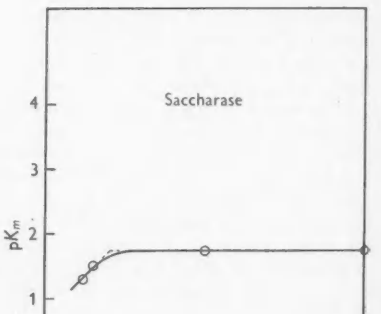

Fig. 6. Effect of pH on pK_m of saccharase. Plotted from data of Josephson (1924).

rabbit arginase. These authors plot values of K_m and draw a smooth curve, but actually all the points between pH's 7 and 9 lie on a horizontal straight line and not on the curve. Their interpretation of the

results, which is no doubt correct, is that in the central range of pH a negatively charged group in the enzyme combines with the positively charged amino group of the arginine, which should give a zero slope; the slope on the alkaline side is then due to the amino group of the substrate (pK about 9) and on the acid side to the group in the enzyme (to which they assign a pK of 7).

Saccharase. In this case the substrate does not ionize. Josephson (1924) states that the affinity of saccharase for sucrose is constant between pH's 4 and 8, but falls off in more acid solution. The experimental points are not sufficiently numerous to draw an accurate curve, but as shown in Fig. 6 they indicate that one of the substrate-binding groups in the enzyme has a pK of about 3. It has been suggested that this group is an amino group, but the pK value makes this unlikely and would correspond better with a carboxyl group. Of course this may not be the only substrate-binding group and the sugar may combine at the same time with other groups which do not ionize, at any rate within this pH range.

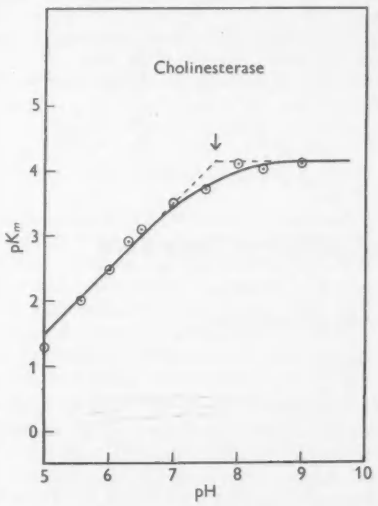

Fig. 7. Effect of pH on pK_m of cholinesterase. Plotted from data of Hase (1952).

Fumarase. The effect of pH on the affinity of pure crystalline fumarase for substrates and inhibitors has recently been studied in detail by Massey. Some complications are introduced by the fact that several of the substances act also as activating anions, but the results, which are being reported separately (Massey, 1953), are of much interest and should throw light on the nature of the active centre of the enzyme and its mode of action.

Cholinesterase. The effect of pH on the action of horse serum on acetylcholine has been studied by Hase (1952), and his results on the K_m have been plotted in Fig. 7. He draws attention to the fact that the tangent of the graph in the more acid region is 1, and ascribes the bend, no doubt correctly, to an ionization of the enzyme with a pK of 7·7. As the substrate is positively charged throughout this pH range, the data suggest a combination $E^- + S^+ \rightarrow ES$ (zero slope) on the alkaline side and $E + S^+ \rightarrow ES$ (+1-unit slope) on the acid side. It may be mentioned, in order to avoid confusion, that this author uses the symbol ϕ_s for our K_m and that he uses the symbol 'K_m' with a different meaning.

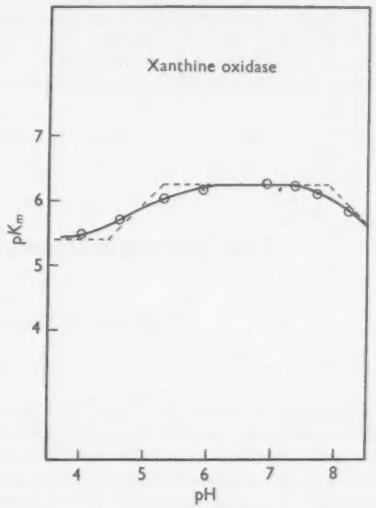

Fig. 8. Effect of pH on pK_m of xanthine oxidase acting on 2-amino-4-hydroxypteridine. Plotted from data of Lowry, Bessey & Crawford (1949). The dotted line shows a possible interpretation.

Xanthine oxidase. Lowry, Bessey & Crawford (1949) have studied the oxidation of 2-amino-4-hydroxypteridine by xanthine oxidase and their data are plotted in Fig. 8. If we assume pK's for the free enzyme (or substrate) of 5·3 and 7·9, and one for the complex of 4·4, the theory would give the curve drawn, which agrees quite well with the experimental points. In the absence of further data, however, this interpretation should not be taken as more than a suggestion.

SUMMARY

It is shown that if the affinities of enzymes for substrates and inhibitors are expressed on a logarithmic scale (pK_s or pK_i) the data can be interpreted by

simple rules. These rules, which are identical in form with those already worked out for oxido-reductions and phosphorylations (Dixon, 1949), enable deductions about the nature of the enzyme-substrate or enzyme-inhibitor combination and the ionization constants of the groups involved to be drawn from the effect of pH upon the affinities.

I am grateful to Dr R. K. Morton for allowing me to quote his unpublished experiments on phosphatase.

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The Determination of Enzyme Inhibitor Constants

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The Michaelis constant, K_m, that is to say the equilibrium constant of the reversible combination of an enzyme with its substrate, is most conveniently determined by Lineweaver & Burk's (1934) method of plotting. The great advantage of this method is that by plotting $1/v$ against $1/s$ it makes it possible to represent the Michaelis equation

$$v = \frac{Vs}{K_m + s} \qquad (1)$$

by a straight line. Here v is the reaction velocity with the substrate concentration s, and V is the maximum velocity obtained at high substrate concentrations. Lineweaver & Burk calculate K_m from the slope of the line and its intercept on the vertical axis, but there is a simpler method which they do not mention, namely to produce the line to the left of the vertical axis, when it will cut the base-line at a point giving $-1/K_m$, as shown in Fig. 1. This is easily shown by putting $1/v = 0$ in the reciprocal form of equation (1), namely

$$\frac{1}{v} = \frac{K_m}{V}\frac{1}{s} + \frac{1}{V},$$

which then gives $1/s = -1/K_m$.

The inhibitor constant, K_i, that is to say the equilibrium constant of the reversible combination of the enzyme with a competitive inhibitor, has hitherto usually been obtained by calculation from the Michaelis equation for a competitive system

$$v = \frac{Vs}{K_m\left(1 + \frac{i}{K_i}\right) + s}, \qquad (2)$$

where i is the inhibitor concentration. For this calculation the effect on the velocity of varying independently both s and i must be determined.

There is, however, a simple graphical method of determining K_i which, as far as the writer is aware, has not been described previously. If $1/v$ is plotted against i, keeping s constant, a straight line will be obtained, and if this is done at two different substrate concentrations s_1 and s_2 the lines will cut one another at a point on the left of the vertical axis, as shown in Fig. 2. This point lies at $-K_i$, which can therefore be read off directly.

The proof is as follows. Each line represents the reciprocal form of equation (2), namely

$$\frac{1}{v} = \frac{K_m}{Vs} + \frac{1}{V} + \frac{K_m}{Vs}\frac{i}{K_i}. \qquad (3)$$

At the point of intersection $1/v$ and i will be the same for both lines, as also will V since the inhibition is competitive. Therefore

$$\frac{K_m}{s_1}+1+\frac{K_m}{s_1}\frac{i}{K_i}=\frac{K_m}{s_2}+1+\frac{K_m}{s_2}\frac{i}{K_i},$$

or

$$\frac{1}{s_1}\left(1+\frac{i}{K_i}\right)=\frac{1}{s_2}\left(1+\frac{i}{K_i}\right).$$

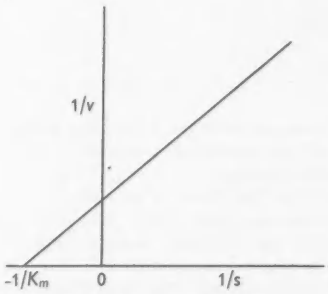

Fig. 1. Determination of K_m.

This can only be true if either $s_1=s_2$ or $i=-K_i$.

If desired, K_m can also be determined from the same plot when K_i has been found, for each line cuts the base-line at a value of i equal to $-K_i(s/K_m+1)$.

Alternatively, if K_m has already been determined by a Lineweaver plot in the absence of inhibitor, it is only necessary to carry out inhibition experiments at one substrate concentration. The intersection point giving K_i lies at a height of $1/V$, as may be seen by putting $i=-K_i$ in equation (3), and this quantity will already have been given by the Lineweaver plot (intersection with the axis for $1/s=0$). It is therefore only necessary to draw a horizontal line at a height of $1/V$, and the point where it intersects the inhibitor line will give $-K_i$. This procedure, however, must only be used for competitive cases.

The case of a non-competitive inhibitor is shown in Fig. 3. Here the lines do not cross, but they meet at a point on the base line which again gives $-K_i$. This is easily seen on putting $1/v=0$ in the reciprocal non-competitive equation

$$\frac{1}{v}=\frac{1}{V}\left(1+\frac{K_m}{s}\right)\left(1+\frac{i}{K_i}\right). \qquad (4)$$

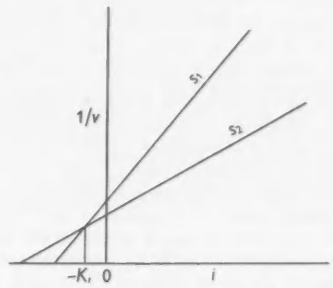

Fig. 2. Determination of K_i (competitive inhibition).

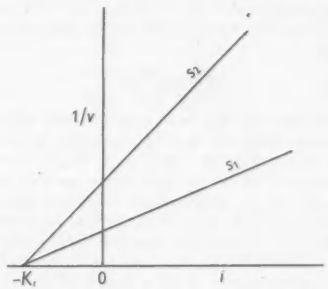

Fig. 3. Determination of K_i (non-competitive inhibition).

SUMMARY

A simple graphical method for determining enzyme inhibitor constants is described.

REFERENCE

Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.

Studies on Fumarase

4. THE EFFECTS OF INHIBITORS ON FUMARASE ACTIVITY

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In recent years much use has been made of competitive inhibitors as tools for investigating the mode of action of enzymes. However, there has been little systematic study of the variation of inhibition with such factors as temperature and pH. Such a study might be expected to throw valuable light on the mechanism of a particular enzymic reaction, since it would be expected to yield information about the thermodynamics of the process and, from the recent theory of Dixon (1953), about the changes in electrical charge accompanying both activation and inhibition. In this paper is described the result of such an investigation with fumarase.

METHODS

The results reported here have been obtained with crystallized fumarase using the method of activity determination already described (Massey, 1953*a*). All the activity values used were initial velocities. The temperature was controlled to within $\pm 0{\cdot}1°$ by means of an apparatus designed by Dr M. Dixon, F.R.S. This device, which comprises a cell holder through which water from a constant-temperature bath is circulated, will be described in detail by Dr Dixon in a later communication. Unless otherwise stated, all the results were obtained in the presence of 0·06 M-phosphate buffers. It has been shown (Massey, 1953*a*) that the initial velocity of the fumarase reaction decreases in the presence of large amounts of phosphate. This seems to be a non-specific effect of ionic strength; accordingly, care had to be taken that the total ionic strength of the reaction mixture was not too high. This limits the accurate determination of the inhibitor constants of weak inhibitors, as the highest concentration at which they can be safely tested, without non-specific salt inhibition, is in the vicinity of 0·1 M. In all cases reported in this paper the total ionic strength has been kept below 0·3. The method used for determining the type of inhibition was that of Lineweaver & Burk (1934) in which the reciprocal of the initial velocity is plotted against the reciprocal of the substrate concentration. The inhibitor constant (K_i) of a competitive inhibitor, which is a dissociation constant of the enzyme-inhibitor complex (EI), was calculated from the Lineweaver & Burk plots by the formula

$$K_i = \frac{[I] \times K_m}{(K_p - K_m)},$$

where $[I]$ is the concentration of inhibitor; K_m is the Michaelis constant and K_p is the apparent Michaelis constant in the presence of the particular concentration of inhibitor used.

RESULTS

The following substances have been tested in this work and are classified into the types of effect which they produce.

Competitive inhibitors. The sodium salts of D-malic, *trans*-aconitic, citric, mesaconic, maleic, adipic, glutaric, succinic, malonic and D-tartaric acids, glycine, and the sodium salt of L-α-hydroxy-β-sulphopropionic acid (the deaminated derivative of L-cysteic acid, L-ethane-1-carboxy-1-hydroxy-2-sulphonic acid).

Non-competitive inhibitors. Thiocyanate, the sodium salt of acetylenedicarboxylic acid.

Non-inhibitory. The sodium salts of acetic, butyric, crotonic, L- and DL-aspartic and acetoacetic acids, the mono- and di-methyl esters of fumarate, and the SH inhibitors, iodoacetate, iodoacetamide, iodosobenzoate, *p*-chloromercuribenzoate and chloroacetophenone.

Table 1 shows the inhibitor constants at pH 6·35 and 23° of compounds which have been found to behave strictly as competitive inhibitors of fumarase.

Table 1. *The effect of structure on inhibition of fumarase at* pH 6·35 *and* 23°

Inhibitor	K_i (molar)
Adipate	$1{\cdot}0 \times 10^{-1}$
Succinate	$5{\cdot}2 \times 10^{-2}$
Glutarate	$4{\cdot}6 \times 10^{-2}$
Malonate	$4{\cdot}0 \times 10^{-2}$
D-Tartrate	$2{\cdot}5 \times 10^{-2}$
Mesaconate	$2{\cdot}5 \times 10^{-2}$
L-α-Hydroxy-β-sulphopropionate	$1{\cdot}65 \times 10^{-2}$
Maleate	$1{\cdot}1 \times 10^{-2}$
D-Malate	$6{\cdot}3 \times 10^{-3}$
Citrate	$3{\cdot}5 \times 10^{-3}$
trans-Aconitate	$6{\cdot}3 \times 10^{-4}$

Within the limits of experimental error the inhibition constants of the reagents tested are the same whether the substrate is fumarate or L-malate. This is taken as evidence that (as might be expected) the same active sites on the enzyme are concerned with the hydration of fumarate as with the dehydration of L-malate.

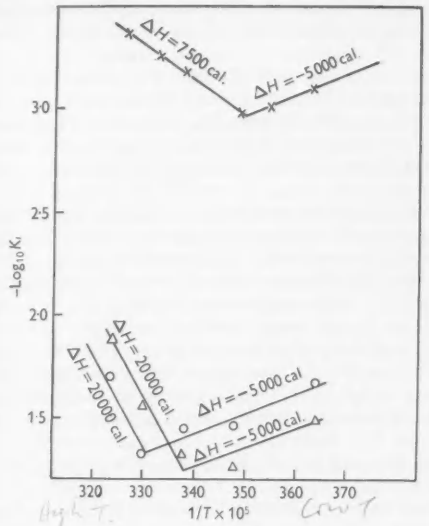

Fig. 1. The effect of temperature on K_i: ×, *trans*-aconitate; △, succinate; ○, malonate.

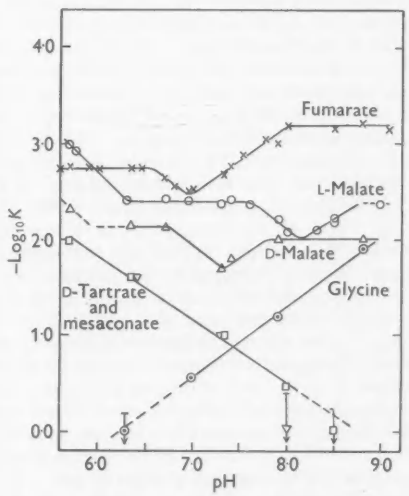

Fig. 2. The effect of pH on K_m and K_i at 23°. K denotes K_m for fumarate and L-malate, and K_i for the other substances. Signs of the type ⊤ ↓ indicate the range of accuracy of the experimental point, the arrow indicating that the value of $-\log_{10} K$ may lie anywhere in the direction of the arrow. ×, Fumarate; ○, L-malate; △, D-malate; □, D-tartrate; ▽, mesaconate; ⊙, glycine.

The effect of temperature on inhibition

The inhibition by all the reagents tested varied with temperature. Fig. 1 shows the effect of temperature on the inhibition constants of *trans*-aconitate, succinate and malonate at pH 6·35. It is seen that at low temperatures the combination of enzyme and inhibitor is exothermic, while at higher temperatures the combination is endothermic. A similar phenomenon occurs with the combination of fumarate and enzyme at acid pH values (Massey, 1953*b*). There, however, the combination of enzyme and substrate was endothermic over the range of temperature investigated (2–35°), but at about 18° there was a changeover from a low heat of formation of the enzyme-substrate complex (*ES*) at low temperatures to a higher heat of formation at higher temperatures.

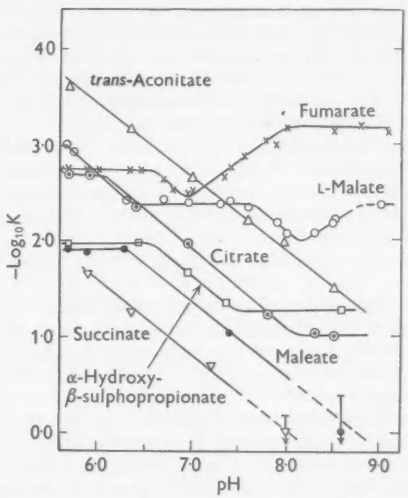

Fig. 3. The effect of pH on K_m and K_i at 23°. K denotes K_m for fumarate and L-malate, and K_i for the other substances. Signs of the type ⊤ ↓ indicate the range of accuracy of the experimental point, the arrow indicating that the value of $-\log_{10} K$ may lie anywhere in the direction of the arrow. ×, Fumarate; ○, L-malate; △, *trans*-aconitate; ⊙, citrate; □, L-α-hydroxy-β-sulphopropionate; ●, maleate; ▽, succinate.

The effect of pH *on inhibition*

A decrease in the inhibition as the pH was shifted toward alkaline values was observed with several inhibitors, and led to a detailed study of the effect of pH on the inhibitor constants of competitive inhibitors of fumarase. Figs. 2 and 3 show the effect of pH at 23° on the dissociation constants (K_i) of a number of inhibitors. These figures also show

for comparison the effect of pH at this temperature on the K_m values for both fumarate and L-malate.

D-Malate is the only inhibitor found to behave in an analogous way to the natural substrates. The dissociation constants of these three compounds with fumarase were found to be independent of pH over a certain range, then with increasing alkalinity to increase, pass through a maximum (corresponding roughly in the case of the two natural substrates to the pH of optimal activity), then to decrease and finally to become once more independent of pH. The K_i values of *trans*-aconitate, tartrate, mesaconate and succinate all continue to increase when the pH is shifted toward alkaline values, until at pH 8·5 *trans*-aconitate is the only one of these compounds to show any appreciable inhibition.

With maleate the inhibition is constant over the pH range 5·7–6·4, then it decreases as the pH is increased. The variation of inhibitory behaviour with pH of citrate and α-hydroxy-β-sulphopropionate is also of a different type. Within the range of phosphate buffers, as the pH is increased their inhibition is at first constant, then decreases and finally becomes independent of pH once more.

The effect of pH on the inhibition by glycine is quite different. At pH 6·35 there is no detectable inhibition, as the pH is made more alkaline, glycine begins to inhibit, and at pH 8·5 the inhibition is quite strong.

DISCUSSION

No explanation of the observed behaviour of inhibition with temperature is obvious at present. The results do demonstrate the care which must be taken in the interpretation of inhibition results. Over a certain temperature range succinate is a more potent inhibitor than malonate, but over another range of temperature the converse is true. The same thing is true of the effect of pH on the activity of some inhibitors. If inhibition constants are determined at only one temperature and one pH, as is often the case, highly erroneous conclusions may be drawn.

The results taken as a whole indicate that the combination of enzyme and substrate or enzyme and inhibitor is largely ionic in nature, or that at least an initial ionic attraction is necessary before combination can occur. Thus a large number of ionized acids ranging in size from C_3 to C_6 can combine with fumarase, and the extent of this combination is influenced greatly by pH. When the ionization of one of the acidic groups is suppressed, then the inhibition, and hence combination of enzyme and acid, is also suppressed. Thus neither the monomethyl ester nor the dimethyl ester of fumarate is acted on by fumarase, nor are they inhibitory. Also crotonate, which resembles fumarate except that one of the carboxyl groups is replaced by a methyl group, does not combine at the active centre. Similarly, aspartic and cysteic acids, which under the pH conditions tested are strongly dipolar at one end of the molecule (and hence may be considered as approaching monoionized acids), do not combine at the active centre. Hence it appears that a two-point attraction of the substrate or inhibitor to the active centre is necessary for combination to occur.

The importance of the ionic attraction of the enzyme and substrate in the formation of a complex is shown also by the inhibition produced by L-α-hydroxy-β-sulphopropionate. This molecule is identical with L-malate except that the carboxyl group further away from the hydroxyl group is replaced by a sulphonic acid group. The fact that it will combine at the active centre indicates that a carboxyl group of L-malate may be replaced by a sulphonic acid group, and combination will still occur. The fact that this compound is not acted on by fumarase is further evidence of the great specificity of this enzyme and is indicative of the sensitive ionic balance that must be required for the catalytic action of the enzyme.

The most obvious ionic attaction would be between the ionized carboxyl groups of the substrate and positively charged groups in the protein. As neither crotonate nor the monomethyl ester of fumarate will cause any inhibition, presumably a two-point attachment of enzyme and substrate or enzyme and inhibitor must occur. However a three-point attraction of such compounds as *trans*-aconitate and citrate may occur, and be responsible for their great affinity for fumarase.

An interpretation of the pH behaviour of the inhibitors and the substrates can be obtained in the light of the theory developed by Dixon (1953). This theory, which is a development of his theory of rH and rP (Dixon, 1950), applies the principles developed in these concepts to the combination of enzyme with substrates and inhibitors. From this theory the ionization constants (pK's) of various groups in the enzyme, substrate or enzyme-substrate complex can be obtained. This information is obtained from the plot of pK_m or pK_i against pH. Such plots are shown in Figs. 2 and 3. A zero slope indicates that the number of charges on ES is equal to the number on the free enzyme plus the number on the substrate; a negative slope of unity means that the free enzyme and free substrate contain one more negative charge than ES; the converse is true for a positive slope. Changes from one slope to another represent ionization constants: a change of the type concave side downward shows a pK value of some group in the free enzyme or the free substrate (or inhibitor); a change of the type concave side upward shows a pK value of some group of ES or EI. An examination of Figs. 2 and 3 shows the

existence with fumarase of all three kinds of slopes, and pK values for both free enzyme and ES and EI.

With fumarate as substrate there is no change of K_m between pH's 5·6 and 6·6. At pH 6·6 there is a changeover to a negative slope which approximates to a one-unit slope. At pH 7·0 this changes over to an approximately one-unit positive slope, which continues until pH 8 where K_m again becomes independent of pH. This means that pH 6·6 is a pK value of either fumarase or fumarate. As the pK values of fumarate are at pH's 3·0 and 4·3 at this temperature, this means that fumarase has a group with a pK value of 6·6 at the active centre. pH 7·0 must represent the pK values of two groups in the enzyme-fumarate complex, as there is a changeover from a negative to a positive slope. Similarly, pH 8 must represent a pK value of a group in the free enzyme as fumarate has no dissociation constant at this pH value.

The change with pH of the K_m of L-malate is similar except that the whole curve is shifted to higher pH by about one unit. Thus the first pK value for the free enzyme occurs at about pH 7·5 and the two pK values for the enzyme-malate complex at about pH 8. Also, another pK value of ES has shown up at pH 6·3, which does not occur with fumarate in the pH range studied, and the second pK value for the free enzyme (which occurs at pH 8 when fumarate is the substrate) has not shown up clearly in the range of phosphate buffers.

Of the inhibitors D-malate is the only one to behave in a similar way to the substrates, that is with positive, negative and zero slopes. The pK values of fumarase determined in the presence of D-malate are at about pH's 6·8 and 8·0; the pK values of EI are at about pH 7·3 and perhaps in the neighbourhood of 6·0.

Citrate and α-hydroxy-β-sulphopropionate show pK values for the free enzyme at pH's 6·0 and 6·6 respectively, and pK values for EI at pH's 8·1 and 7·5 respectively. However, they give only one pK value each for free enzyme and EI, compared with at least two of each shown with fumarate, L-malate and D-malate.

trans-Aconitate does not give any pK value of either free enzyme or EI over the whole pH range investigated (5·7–8·5). Similar results were obtained with D-tartrate, mesaconate and succinate. With these last compounds, however, the possibility of a pK value of the free enzyme or EI is not ruled out owing to the difficulty of obtaining an accurate value for K_i when the inhibition is very small, as there is a limit to the concentration of inhibitor which can be used.

The results with maleate indicate a pK value of either fumarase or maleate at pH 6·4. Maleic acid has in fact a pK of about this value.

Glycine behaves in the opposite manner to *trans*-aconitate, etc., giving a positive slope of almost unity, indicating that the fumarase-glycine complex contains one more negative charge or one less positive charge than the free enzyme and glycine.

As both substrates and inhibitors yield different pK values for the free enzyme it is probable that the combination of the enzyme with these inhibitors or substrates alters the ionization constants of groups in the protein. This could conceivably occur by combination at two different sites in the enzyme, at the active centre and at some other site. The combination at the other site might quite well modify the ionization constants of groups at the active centre, since the ionization of these groups is likely to be influenced by the proximity of other charged groups. It is difficult, however, to visualize how *trans*-aconitate, etc., could produce such a large shift in the pK values. Nevertheless, evidence for such a dual reaction does exist, since citrate, which has been shown here to behave as a competitive inhibitor, will cause considerable activation of salt-free fumarase (Massey, 1953*a*).

Such a duality of effect would account for the different pK values of the enzyme found with different compounds, and as will be shown later, it might also account for the different pH optima of fumarase with its two substrates. If such a reaction does occur, then what has been referred to throughout as free enzyme really means enzyme which is not combined at the active centre.

The possibility also exists that if substrates and inhibitors combine at groups other than the active centre, this secondary combination may affect the values of K_m and K_i. In this case the measured values of K_m and K_i would not be the true values. Such an effect might contribute to the different values found under different conditions for the dissociation constants of groups in the 'free enzyme'.

The mechanism producing the observed changes in electrical charge associated with formation of ES or EI remains obscure. The inhibition by glycine suggests that glycine may be inhibiting by ionic attraction of its charged carboxyl group to one of the positively charged groups in the protein responsible for attraction of enzyme to substrate, and by attraction of its charged amino group to an acidic group at or close to the active centre. Presumably such a two-point attraction must occur. If glycine were to be attracted only by its carboxyl group, such a method of attraction should also apply to crotonate and the monomethyl ester of fumarate. However, this does not seem to occur, as no inhibition could be detected by these compounds. According to Dixon's theory the glycine-fumarase complex contains one more negative or one less positive group than the 'free' enzyme and free

glycine. If the proposed acidic group in the protein were a weak acidic group such as a sulphydryl or a phenolic group, this would be unionized over most of the pH range investigated. However, bringing the strong positive charge of the amino group of glycine into the proximity of this potential negative group would be expected to cause it to ionize; the fumarase-glycine complex would then contain one more negative charge than the 'free' enzyme and glycine. However, no such positive group exists in the substrates to account for the similar slopes found with fumarate and malate.

If such an acidic group does exist at the active centre it is more likely to be a phenolic than a sulphydryl group for, contrary to the report of Laki (1941), no evidence could be obtained that crystallized fumarase contains a sulphydryl group essential for its activity. Iodoacetate, iodoacetamide, iodosobenzoate, *p*-chloromercuribenzoate and chloroacetophenone have all been tested on fumarase and found to be without effect.

If, as the evidence indicates, there is a secondary interaction of substrates and inhibitors which affects the ionization constants of groups at the active centre, it is obviously impossible to draw any rigorous conclusions about the chemical nature of the groups whose pK values have been determined. However, some tentative conclusions can be made. Fumarate and several inhibitors show pK values of some group in the 'free' enzyme about 6·0–6·7. The glyoxaline group of histidine is the only group known in proteins which has a pK value in this region, so that it is likely that this group occurs at the active centre of fumarase.

The existence of a glyoxaline group at the active centre is consistent with the changes of charge associated with formation of *ES* or *EI* complexes. At low pH values the glyoxaline group would be fully ionized and the attraction of substrate (or inhibitor) by a basic group in the enzyme adjacent to the glyoxaline group should not have any influence on the ionization of this glyoxaline group. However, at pH values above its pK the glyoxaline group would be becoming unionized. The 'neutralization' of an adjacent basic group would then tend to cause a re-ionization of the glyoxaline group; the *ES* or *EI* complex would then possess one more positive charge than the 'free' enzyme and substrate (or inhibitor).

These conclusions suggest the possibility of a grouping of charges at the fumarase active centre as shown in Fig. 4.

Such a grouping of charges might provide an explanation of the sequence of events associated with the enzymic reaction. Considering first the reaction starting with fumarate as substrate, fumarate is probably orientated at the active centre primarily by ionic attraction of its charged carboxyl groups to the positively charged groups 1 and 4 of the protein. This would bring the two α-carbon atoms of fumarate in proximity to the proposed acidic and basic groups 2 and 3. When both of these groups 2 and 3 are ionized they might be expected to exert a polarizing effect on the two α-carbon atoms. Thus the carbon atom α_1 would become electron-poor and the carbon atom α_2 would become electron-rich. This might be followed by the addition of water between the polarized carbon atoms, either as H^+ and OH^-, or as the dipolar molecule of water, giving the fumarase-L-malate complex.

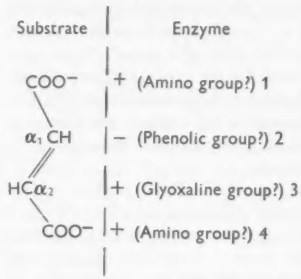

Fig. 4. A possible structure of the active centre of fumarase.

A similar mechanism might apply for the conversion of the L-malate complex to the fumarate complex. If the carbon atom which becomes electron-rich were the one to which the hydroxyl group is attached, the inherent electron-rich nature of the hydroxyl group should cause this to be split off by electrostatic repulsion, and a double bond between the two α-carbon atoms would be formed. From this hypothesis, the pH of optimal activity would be expected to be where the polarizing effects of groups 2 and 3 are equal, that is, where they are equally ionized. From Figs. 2 and 3 it can be seen that two groups in *ES* have pK values which are identical. Thus the enzyme-fumarate complex has two pK values at 7·0, and the enzyme-L-malate complex has two pK values at 8. These pK values are approximately the same as the pH values of optimal activity for the two substrates (Massey, 1953*a*). Hence the two pK values of each enzyme-substrate complex may represent the pK values of the proposed groups 2 and 3 when the enzyme-substrate complex is formed, that is, the combination of enzyme and substrate would shift the pK of the glyoxaline group 3 to higher values, and the dissociation constant of (the phenolic?) group 2 to lower values.

SUMMARY

1. The following compounds have been found to be competitive inhibitors of fumarase: the sodium salts of D-malic, *trans*-aconitic, citric, mesaconic, maleic, adipic, glutaric, succinic, malonic and

D-tartaric acids, glycine and the sodium salt of L-α-hydroxy-β-sulphopropionic acid.

2. Thiocyanate and the sodium salt of acetylenedicarboxylic acid were found to be non-competitive inhibitors.

3. The following compounds were found to be without effect on fumarase: the sodium salts of acetic, butyric, crotonic, L- and DL-aspartic, and acetoacetic acids, the mono- and di-methyl esters of fumarate, and the SH inhibitors, iodoacetate, iodoacetamide, iodosobenzoate, *p*-chloromercuribenzoate and chloroacetophenone.

4. The inhibition of all the competitive inhibitors was dependent on the temperature. Of the three inhibitors investigated in detail at low temperatures the combination of fumarase and inhibitors was found to be exothermic, while at higher temperatures the combination was found to be endothermic.

5. Inhibition by all the competitive inhibitors tested was markedly dependent on pH. The variation of the inhibitor constants and of the Michaelis constants with pH has been studied in detail, and the results are discussed in the light of the theory of Dixon (1953).

6. A tentative hypothesis of the structure of the active centre and the sequence of reactions which may accompany the reversible hydration of fumarate to L-malate is suggested on the basis of these results.

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Absorption of Immune Globulin by the Young Lamb after Ingestion of Colostrum

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The role of colostrum in promoting the well being of the young ruminant has been established mainly by work on the cow. It has been shown that these animals depend on the ingestion of colostrum soon after birth and the absorption of immune globulins from this secretion for a passive immunity which greatly favours their chances of survival of the critical first few weeks of their life (Smith & Little, 1922; Orcutt & Howe, 1922). That a similar situation obtains in the sheep has been shown in three different ways; the immunological studies of Mason, Dalling & Gordon (1930) and Schnieder & Szathmary (1939); the work on the sodium sulphate salted-out protein fractions of Earle (1935) and the electrophoretic examinations of Charlwood & Thomson (1948). In the present work these three methods have been combined in a study of the changes in the serum proteins of suckling lambs and extended to a series of lambs receiving colostrum after various periods of delay. In relation to these changes, the existence and nature of any proteinuria in the young lambs has been investigated.

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METHODS

Salted-out nitrogen fractions. Serum protein, globulin and albumin N fractions were estimated by difference from the total N, 'globulin-free' N and non-protein N contents; these were determined by micro-Kjeldahl analysis of the following solutions representing a dilution of 1 in 21 of the serum sample: a dilution in normal saline, a globulin-free filtrate obtained after precipitation with saturated $MgSO_4$ at 20° overnight, and a protein-free filtrate obtained after precipitation with trichloroacetic acid (5 g./100 ml.). Colostrum N fractions were obtained by analysing a suitable dilution in saline in the same way as serum with the following addition: casein N was obtained by including the analysis of a casein-free filtrate prepared by acidification of a dilution in saline with acetic acid.

Electrophoretic examination of the sera. This was carried out in the Tiselius apparatus at 4° using phosphate buffer at pH 8·0 and ionic strength 0·2 and a protein concentration of about 2 g./100 ml. The percentage composition was determined from the diagrams by projection, extrapolation and planimetry. As considerable difficulty was experienced in analysing the individual components, the method of extrapolation employed by Dalgarno, Godden & McCarthy (1950) was used throughout. The albumin and salt boundaries were completed. The slowest component, probably corresponding to the γ-globulin of human serum was then extrapolated and called component 2. The area between this component and the albumin was not subjected to further analysis but was measured as a single component and termed component 1.

Antibodies. Antibodies in colostrum, serum and urine were estimated by the limiting dilution of the agglutinin test. At first the tests were carried out from a lower limit of 1 in 25, but later, samples which were negative at this limit were examined on a lower range from a dilution of 1 in 2.

Urine examination. This consisted of qualitative tests for protein by sulphosalicylic acid precipitation and by heat coagulation (with addition of acetic acid). Globulin was detected by precipitation with half-saturated $(NH_4)_2SO_4$ and with saturated $MgSO_4$. These tests were made comparative by an arbitrary grading of the degree of precipitation as a trace, +, + + and + + +. Antibodies were tested for in the lower range of dilutions, that is from a dilution of 1 in 2.

RESULTS

The present observations were made on Cheviot, Blackface and half-bred lambs over a period of two lambing seasons. The ewes which gave birth to them were immunized with *Salmonella typhosa* antigen. The first injection was given 21 days before the expected lambing date. After birth, some lambs were allowed to suck the ewe in the normal way, some were bottle-fed with cows' milk for varying periods before colostrum feeding, while others never received colostrum. The lambs which received colostrum after a delay, did so, either by returning to the unsucked dam when the delay was short, or by being fostered on to another ewe which had recently lambed but not suckled, or by ingesting a single pint of stored colostrum. The relations and treatments of these animals are given in Table 1.

Observations were made on 15 ewes and 17 lambs. Seven lambs ingested colostrum at birth, six after delays of 12, 24, 29 and 48 hr. and 3 and 9 days, respectively, and four were deprived of colostrum.

The serum and colostrum of the ewes at lambing were examined for salted-out nitrogen fractions and antibodies. In two ewes, the colostrum was further examined at various intervals after lambing. In two other ewes it was also examined before lambing took place. The results presented for the ewes, Table 2, have been limited to thirteen relating to those given for the lambs, Table 3.

The lambs' serum was also examined by these two methods and in addition by electrophoretic analysis. Samples were taken at the following times: birth, 2–3 days, 7–10 days and at 5–6 weeks after birth. The results, Table 3, in the case of lambs ingesting colostrum at birth, have been limited to four typical cases. The urine of twelve lambs was examined for a few days after birth and ingestion of colostrum, Table 4.

The composition of colostrum, Table 2, showed at lambing protein nitrogen ranging from approximately 1 to 4 g./100 ml. and titres from 1 in 1000 to 1 in more than 10 000. The subsequent rapid change in composition is illustrated by the data included for ewes 232 and 255, Table 2.

The observations on colostrum before lambing were made on ewes 13 and 22, Table 2. The colostrum of ewe 13 obtained 3 days before lambing resembled the richer samples in its nitrogen distribution and agglutinin titre. The other, from ewe 22, 12 days

Table 1. *Lambs, dams and treatments*

Kept on the ewe			No colostrum		
Lamb no.	Ewe no.	Breed	Lamb no.	Ewe no.	Breed
512	178	Cheviot	452	255*	Blackface
518	191	Cheviot	415	232	Blackface
672	46	Half-bred	514	190	Cheviot
673	42	Half-bred	674	28	Half-bred
675	28	Half-bred			
678	51	Half-bred			
686	22	Half-bred			

Colostrum delayed				
Lamb no.	Ewe no.	Breed	Delay	Method of receiving colostrum
680	53	Half-bred	12 hr.	Returned to dam
683	13	Half-bred	24 hr.	Returned to dam
682	26	Half-bred	29 hr.	Given 200 ml. colostrum from ewe 53
519	191	Cheviot	48 hr.	Fostered on to ewe 172
677	51	Half-bred	3 days	Fostered on to ewe 37
513	178	Cheviot	9 days	Fostered on to ewe 190

* Not immunized.

Table 2. *Nitrogen distribution and titres of ewes' serum and colostrum*

		Serum				Colostrum				
		N mg./100 ml.				N mg./100 ml.				
Ewe no.	Days from lambing	Protein	Globulin	Albumin	Titre	Protein	Casein	Globulin	Albumin	Titre
13	3 before	—	—	—	—	3767	645	2732	390	7500
	0 after	1251	590	661	500	2635	813	1347	475	>10000
22	12 before	—	—	—	—	5275	755	4045	475	<25
	0 after	1095	589	506	500	2706	210	2157	339	1000
26	0 after	1082	568	514	2500	4136	1122	2288	726	2500
28		1036	555	481	500	3390	1390	1565	435	1000
37		1241	725	516	2500	1183	613	300	270	1000
51		1015	573	442	10000	2047	879	838	330	2500
53		1170	670	500	1000	2174	875	1096	203	2500
172		900	430	470	1000	3100	1245	1317	538	2500
178		982	486	496	2500	2980	804	1817	359	>10000
190		928	442	486	5000	2990	1410	1186	394	>10000
191		900	430	470	1000	2677	1357	955	365	2500
232	0 after	1046	442	604	5000	2956	1070	1473	413	10000
	3 after	—	—	—	—	1126	734	253	139	2500
	9 after	—	—	—	—	697	588	41	68	250
	36 after	1213	568	645	1000	687	566	56	65	<25
255	0 after	904	354	550	—	3742	730	2445	567	—
	2 after	—	—	—	—	1191	756	277	158	—
	7 after	—	—	—	—	786	629	66	.91	—
	40 after	1242	661	581	—	859	696	63	100	—

before lambing was most concentrated containing 5 g./100 ml. protein nitrogen of which 77 % was globulin nitrogen but its titre was less than 1 in 25. However, the colostrum from the same ewe at lambing had a titre of 1 in 1000. This suggests that the antibodies may be secreted in the colostrum over a relatively short period immediately before lambing. Mason *et al.* (1930) found antitoxin to lamb dysentery present 12 days before lambing, but their ewes had been immunized throughout pregnancy.

The changes in the lambs' serum proteins. The changes shown in Table 3 and Fig. 1 illustrate the effects of the post-natal occurrence and subsequent cessation of colostrum absorption. The degree to which they do so varies with the method of analysis.

The analysis of globulin nitrogen by salt precipitation showed a marked temporary increase in both absolute and relative concentrations after ingestion of colostrum, when this took place at birth or after 12 hr. delay, inconclusive changes when the delay was 24, 29 and 48 hr. and a gradual increase in absolute but not relative concentration over the period of observation when the delay was longer or when no colostrum was given.

The electrophoretic results showed similar trends in protein composition, but in addition an increase in the proportion of globulin when the delay was 24 hr. The approximate average values of the relative globulin concentrations were 21 % at birth, 55 % a few days after absorption of colostrum and 33 % 5 or 6 weeks later. These changes were mainly due to the globulin referred to as component 2. Its corresponding values were 5, 31 and 11 % respectively. Component 1 remained at about 22 % throughout. By the time the lambs were 5 or 6 weeks old there was little difference in their serum globulin determined by either method whether they had shown absorption or not.

The agglutinin titre clearly showed absorption of globulin in the lambs receiving colostrum up to 29 hr. but not after 48 hr. or more delay. The titres of animals showing absorption rose from negligible values at birth to between 1 in 125 and 1 in 5000 after ingestion of colostrum and fell to quite low values 5 or 6 weeks later.

Comparison of the results obtained on the serum of lambs 512, 518, 678 and 686 (Table 3) with those obtained on the corresponding ewes, 178, 191, 51 and 22 respectively (Table 2) shows that colostrum ingestion and absorption had given to the lamb for the time being a globulin-nitrogen content and an agglutination titre comparable with that of its dam. The lambs serum taken at varying periods after birth is compared with that of the dam at birth only since data on these ewes' serum at corresponding post-natal periods was not obtained. 5 or 6 weeks later the lambs' globulin nitrogen was much less and its titre, as expected, quite small compared with its dam. During this period the lambs' albumin nitrogen rose to a level comparable to that in the dam.

The relative globulin concentration of the lambs' serum has been calculated from the salt precipitation results and included in Table 3. Comparison with the electrophoretically determined globulin shows that they have the same general trends but

Table 3. *Examination of lambs' serum*

Lamb no.	Delay	Age (days)	Nitrogen distribution (mg./100 ml.) Protein	Globulin	Albumin	Titre	Percentage composition: By electrophoresis Albumin	Component 1	Component 2	Globulin	By $MgSO_4$ pptn. globulin
						Kept on the ewe					
512	—	0	739	270	469	<25	73·8	22·0	4·2	26·2	37·2
		2	785	421	364	2500	34·6	22·6	42·8	65·4	54·2
		12	1030	492	538	2500	52·3	27·7	20·1	47·7	48·5
		33	965	353	612	500	—	—	13·5	—	37·4
518	—	2	909	476	433	1000	49·0	23·7	27·3	51·0	53·2
		10	936	313	623	250	53·5	28·0	18·5	46·5	34·1
		36	882	248	634	50	63·2	20·1	16·7	36·8	28·8
678	—	0	713	269	444	<2	76·3	21·1	2·6	23·7	38·3
		4	953	456	497	500	50·7	24·9	24·4	49·3	48·7
		10	920	352	568	125	—	—	—	—	39·2
		33	1077	488	589	<25	68·1	25·3	6·6	31·9	46·0
686	—	¼	878	481	397	250	53·9	20·9	25·2	46·1	55·5
		2½	—	—	—	500	35·6	23·0	38·4	61·4	—
		10	982	445	537	1000	51·6	24·5	23·9	48·4	46·2
		51	935	294	641	<25	—	—	—	—	32·2
						Colostrum delayed					
680	12 hr.	0	801	291	510	<2	—	—	—	—	37·0
		¾	1029	542	487	2500	40·7	24·5	34·8	59·3	53·5
		10	1042	502	540	250	51·6	25·7	22·7	48·4	49·0
		33	847	315	532	125	74·2	14·6	11·2	25·8	38·0
683	24 hr.	¼	787	316	471	<2	72·8	21·1	6·1	27·2	40·8
		3¼	893	367	526	1000	61·9	27·0	11·1	38·1	41·8
		10	752	278	474	50	62·2	28·0	8·8	36·8	37·7
		29	762	270	492	50	69·1	23·3	7·6	30·9	36·2
682	29 hr.	0	717	289	428	16	—	—	—	—	41·2
		10	860	380	480	1000	—	—	—	—	45·0
		31	812	303	509	125	—	—	—	—	38·0
519	48 hr.	2	597	182	415	<2	60·4	26·7	12·9	39·6	31·2
		5	715	265	450	<25	64·9	—	—	35·1	38·0
		10	759	149	610	<2	65·9	27·4	6·7	34·1	20·0
		36	929	344	585	<2	64·3	23·4	12·3	35·7	37·7
677	3 days	0	677	235	442	<2	—	—	6·2	—	35·3
		3	—	—	—	<2	71·4	23·8	4·8	28·6	—
		5	620	219	401	<2	—	—	—	—	36·2
		10	792	249	543	<2	68·5	23·4	8·1	31·5	32·0
		33	785	283	502	<2	71·7	18·5	9·7	28·2	36·6
513	9 days	0	529	194	335	<25	—	—	—	—	37·2
		9	757	262	495	<25	61·4	34·3	3·9	38·6	35·3
		12	812	228	584	<25	59·2	34·1	6·7	40·8	28·8
		33	792	241	551	<25	58·1	31·7	10·2	41·9	31·2
						No colostrum					
452	—	0	731	297	434	—	—	—	—	—	41·4
		2	697	286	411	—	—	—	—	—	41·7
		7	741	309	432	—	—	—	—	—	42·3
		40	898	329	569	—	—	—	—	—	37·2
415	—	0	783	265	518	<25	—	—	—	—	34·5
		3	805	255	550	<25	—	—	—	—	32·4
		10	745	242	503	<25	—	—	—	—	33·2
		36	902	344	558	<25	—	—	—	—	38·8

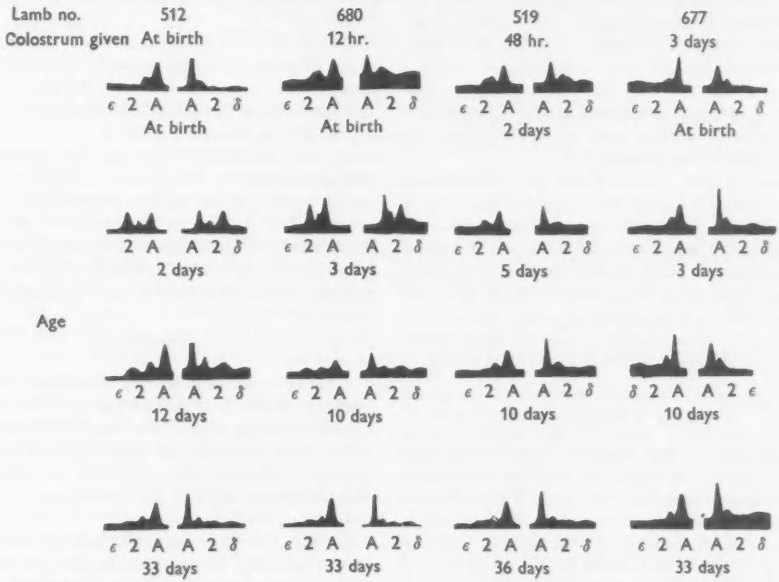

Fig. 1. Electrophoretic patterns of lambs' sera in phosphate buffer at pH 8·0 and ionic strength 0·2. The lambs were given colostrum after various periods of delay and bled at different ages.

Table 4. *Examination of lambs' urine*

Lamb no.	Delay	Age (days)	Protein	Globulin	Titre
		Kept on the ewe			
672	—	1	+ + +	+ +	128
		2	+	+	16
		3	tr.*	tr.	8
		4	tr.	tr.	<2
673	—	1	+ +	+	32
		2	+	tr.	32
		3	tr.	tr.	8
		4	tr.	tr.	2
		10	tr.	tr.	<2
675	—	1	+ + +	+ +	16
		2	+ + +	+ +	4
		3	tr.	tr.	<2
686	—	¼	+ + +	+ + +	32
		1¼	+	+	8
		2½	tr.	tr.	<2
		No colostrum			
514	—	0	tr.	tr.	–
		3	tr.	tr.	–
674	—	1	tr.	tr.	2
		2	–	–	<2

Lamb no.	Delay	Age (days)	Protein	Globulin	Titre
		Colostrum delayed			
580	12 hr.	¼	–	–	<2
		1¼	+ + +	+	32
		2¼	+	+	8
		3¼	tr.	tr.	<2
683	24 hr.	½	–	–	<2
		1½	+	tr.	16
		2½	+ + +	+	32
		3½	+	+	32
		10	tr.	tr.	<2
682	29 hr.	¼	+ + +	+ + +	–
		1¼	+	tr.	16
		2¼	tr.	tr.	8
		3¼	tr.	tr.	<2
519	48 hr.	1	–	–	–
		2	tr.	tr.	–
		3	tr.	tr.	–
677	3 days	½	–	–	<2
		4½	–	–	<2
513	9 days	5	–	–	–
		9	tr.	tr.	–
		10	tr.	tr.	<2
		12	tr.	tr.	<2

* tr. = trace.

are by no means in good or regular agreement. In only eleven out of twenty-eight cases the agreement was within ± 10 %. This may be explained in part by an observation of Jager, Schwartz, Smith, Nicherson & Brown (1950) that too vigorous shaking of serum saturated with magnesium sulphate before filtration may seriously affect the results by protein denaturation.

Results on lambs' urine. These are reported in Table 4 and appear to provide a convenient index of the time of ingestion of colostrum. Proteinuria was marked when ingestion took place at birth and up to 29 hr. after birth; proteinuria was absent or only slight when colostrum was received after 48 hr. or not at all. The proteinuria appeared to include globulin and to be accompanied by an appreciable agglutinin titre. These effects became negligible in a few days.

DISCUSSION

The experimental data suggest that the changes observed in the lamb serum indicate the absorption of immune globulin from colostrum. These changes, together with those in the urine, show that ingestion of colostrum by the lambs up to some time between 29 and 48 hr. after birth leads to the absorption of immune globulin and the appearance or increase of a slow-moving electrophoretic component referred to as component 2. This finding may be compared with that obtained by Hansen & Phillips (1947) on the calf. Their electrophoretic studies showed absorption of gamma globulin at 24 but not at 42 hr. after birth.

The urine tests showed that the kidney of the newborn lamb appears to possess an unusual permeability, which persists for at least the same period after birth as that observed in the alimentary canal.

The permeability or otherwise of the different types of placentae has usually been explained in terms of the number of layers of tissue concerned. The present results indicate that this may not be so important as the properties of a particular layer since the impermeability of the placenta is in marked contrast to the permeability of the numerous tissue layers of the udder, alimentary canal and kidney. The results also show that the placental impermeability is not absolute, as sometimes a small amount of component 2 and a discernible agglutinin reaction could be obtained in the lambs' serum at birth (Table 3).

SUMMARY

1. Immunological, salt-precipitation and electrophoretic methods of investigation have been combined in a study of (*a*) the changes in serum proteins of lambs on ingestion of colostrum at birth, (*b*) the effect of delaying the ingestion of colostrum on these changes and (*c*) the occurrence of any proteinuria in this connexion.

2. The results show that there is an increase in serum globulin largely due to one electrophoretic component and an appearance of antibody, when colostrum is given up to 29 hr. but not at 48 hr. or more after birth.

3. Following the ingestion of colostrum the lambs showed a marked proteinuria, characterized by the presence of globulin and antibody.

We wish to acknowledge our indebtedness to Mr G. Anderson for technical assistance, to Mr W. Thomson and staff for management of the ewes and lambs and Mr R. Cook for the determination of agglutinin titres.

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The Production of Fatty Acids by a Gram-negative Coccus

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We have isolated from the rumen of the sheep an anaerobic Gram-negative coccus which ferments glucose, fructose, maltose, mannitol, sorbitol and DL-lactate with the production of hydrogen and carbon dioxide, acetate, propionate, *n*-butyrate, *n*-pentanoate and *n*-hexanoate (Elsden, Gilchrist, Lewis & Volcani, 1951). Since it is our intention to discuss the taxonomic position of this organism in a separate publication we propose, for the purpose of this communication, to refer to the organism as LC. When LC is grown on glucose the fatty acids containing an even number of carbon atoms, acetate, *n*-butyrate and *n*-hexanoate predominate; whereas during growth on lactate there is a higher proportion of propionate and *n*-pentanoate. The metabolism of this organism was of interest to us in view of the formation of significant amounts of *n*-pentanoate and *n*-hexanoate. As far as is known the only bacteria which produce fatty acids of the *n*-series higher than *n*-butyrate are *Clostridium kluyveri* (Barker & Taha, 1942), *Rhodospirillum rubrum* (Kohmiller & Gest, 1951) and LC (Elsden *et al.* 1951).

Bornstein & Barker (1948) showed that when *Cl. kluyveri* was grown upon ethanol plus either acetate, propionate or *n*-butyrate, the predominant fatty acids formed were *n*-butyrate and *n*-hexanoate, *n*-pentanoate or *n*-hexanoate respectively; in addition, traces of *n*-heptanoate were produced during growth upon ethanol and propionate. Using cell-free preparations of *Cl. kluyveri*, Stadtman & Barker (1949) showed that ethanol was oxidized in the presence of orthophosphate to acetyl phosphate; and this compound was considered to combine with either acetate, propionate or *n*-butyrate to form an intermediary which was reduced to the corresponding fatty acid of the *n*-series containing two more carbon atoms. Subsequent work by Stadtman (1952) on the phosphotransacetylase of *Cl. kluyveri* has made it probable that acetylcoenzyme A (Lynen, Reichert & Rueff, 1951) and not acetyl phosphate is the active form of acetate in this synthesis, though there is as yet no direct proof. The mechanism of the synthesis of fatty acids by *R. rubrum* was not investigated by Kohmiller & Gest (1951).

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In this paper we report the results of our investigations of the synthesis of fatty acids from pyruvate by washed suspensions of LC.

METHODS

Growth media and preparation of washed suspensions. The strain LC 1 was used for these experiments. It was maintained on the following medium: 0·03 M-phosphate buffer (sodium salts) pH 7·0, 1·4% (v/v) of 70% (w/v) sodium lactate (Hopkin and Williams), 0·4% (w/v) Difco yeast extract, 0·05% (w/v) NH_4Cl, 0·02% (w/v) $CaCl_2$, 0·02% (w/v) $MgCl_2$, 0·03% (w/v) thioglycollic acid and 0·1% (w/v) Davis New Zealand agar; the pH was adjusted to 7·0 before autoclaving. The organism was subcultured every 10 days. To prepare an inoculum for mass culture, a tube of the above medium, which contained in addition roughly half its volume of Robertson's meat but no agar, was inoculated with a few drops of a stock culture and incubated until there was a vigorous production of gas (4–8 hr.). The contents of the tube were then transferred, with sterile precautions, to a 250 ml. round, flat-bottomed flask containing 200 ml. of the liquid medium but no Robertson's meat. Anaerobiosis was essential so the flasks were incubated at 37° in a MacIntosh and Fildes anaerobic jar under an atmosphere of H_2 containing 5% CO_2; alternatively, a desiccator, filled with the same gas mixture and containing a capsule of palladized asbestos, was used. After incubation for 20–24 hr. the culture was filtered through glass wool and harvested on the centrifuge. Except where otherwise stated, the organisms were washed twice with and suspended in 0·1 M-phosphate buffer pH 6·5 which had been boiled to remove O_2 and to which 50% (w/v) Na_2S, $9H_2O$ had been added to a final concentration of 0·03% (w/v). The suspensions contained 0·2–1·0 mg. total N/ml. To prevent loss of activity due to exposure to air a slow stream of H_2 was passed through the suspension until it was used.

Manometric methods. The reactions were carried out in Warburg manometer vessels: the main compartment usually contained 2 ml. of the suspension and the substrates were added from the side arms after an equilibration period. When H_2 output was measured the centre wells contained 0·2 ml. 20% (w/v) KOH to absorb any CO_2 produced.

In some experiments it was necessary to determine both H_2 and CO_2 in the same manometer, and the following modification of the Dickens & Šimer (1930) first method was found to be suitable. Conventional double side-bulb cups replace the more complicated cups designed by these authors. The bacterial suspension was placed in the main compartment and in one side bulb was placed 0·2 ml. of CO_2-free, 2 N-NaOH, plus a strip of filter paper folded concertina-wise. Initially we used $Ba(OH)_2$ but we found that this was less efficient than NaOH due, it is thought, to the

formation of a film of barium carbonate over the surface of the filter paper. The second side bulb contained 0·4 ml. 4N-H_2SO_4. The substrate was added from a Keilin dangling tube (Keilin, 1929). The centre well must be sufficiently tall to carry the Keilin tube and, at the same time, placed eccentrically to facilitate manipulation. This involves no radical alteration in the design of Warburg cups.

After the equilibration period, the Keilin tube was dislodged and the experiment allowed to proceed. Experiment showed that the rate of absorption of CO_2 by alkali placed in the side bulb is not quite so rapid as when it is placed in the centre well, but it is adequate for most purposes. At the end of the experiment the acid was added from the side bulb to release the bound CO_2 and the manometers were shaken until no further pressure change occurred, i.e. until all the liberated CO_2 had been absorbed by the alkali. The difference in pressure between the initial reading and that recorded after the absorption of all the CO_2 is due to the production or absorption of gas other than CO_2.

Where, as in our experiments, the production of two gases is being measured, or if a very large amount of CO_2 is expected, it is advisable to expel some gas, to allow the measurement of the CO_2. This having been done, the acid contents of the main compartment are thoroughly mixed with the alkali plus filter paper contained in the side bulb. It is essential for the success of the method to ensure complete acidification of the CO_2 absorbent; and we have found it prudent to repeat the mixing procedure once the pressure has become constant. The pressure developed on acidification is due to the total CO_2 in the system. This is made up of three components, namely, the CO_2 introduced with the suspension, the CO_2 produced during the equilibration period and that formed during the course of the experiment. An estimate of the initial CO_2 is obtained by setting up an additional manometer and acidifying at the end of the equilibration period. This method has been found satisfactory both for the estimation of H_2 and CO_2 produced during fermentation and also for the measurement of the O_2 uptake and CO_2 production by respiring bacterial suspensions.

Analytical methods

Total nitrogen. This was determined by the Kjeldahl method as modified by Chibnall, Rees & Williams (1943) and the ammonia, which was distilled off in the apparatus of Markham (1942), was trapped in the boric acid reagent of Conway & O'Malley (1942) and titrated with standard acid from a microburette.

Pyruvic acid. Estimations were carried out by a modification of the method of Friedeman & Haugen (1943) introduced by Lichstein & Umbreit (1947); an EEL (Evans Electroselenium Ltd., Harlow, Essex) photoelectric colorimeter with a blue filter was used.

Volatile fatty acids. A 2 ml. sample was acidified with 1 ml. 5N-H_2SO_4, and the volatile fatty acids distilled in the apparatus of Markham (1942). The distillate was aerated with CO_2-free air for 3 min. before titrating with CO_2-free NaOH approx. 0·02N using phenol red as the indicator.

A paper-chromatographic method was employed for the identification of the acids present and the procedure used was based on those suggested by Brown (1950), Brown & Hall (1950) and Hiscox & Berridge (1950). Whatman no. 1 paper was used and the chromatograms were developed by the ascending method for 24–30 hr. with *n*-butanol saturated with 1% (w/v, aq.) ethylamine. The paper was dried for 2 hr. at room temperature and sprayed with 0·04% (w/v) ethanolic bromocresol green. On standing in air for 2–4 hr. the fatty acids become visible as greenish blue spots on a yellowish green background.

The mixtures of volatile acids with which we were dealing were complex, and the amounts smaller than could be handled by any of the existing methods, and we were particularly fortunate that, at about this time, James & Martin (1952) introduced the liquid-gas partition chromatogram. Our procedure followed closely the directions of the authors save that we used an Agla (Burroughs Wellcome Ltd.) syringe to titrate the acids as they emerged from the column. Since the total amount of acid we had available was small, we used the method to determine the proportions of the constituent volatile acids in the mixture and, knowing the total volatile acid content, were able to calculate the amounts of each acid present. Using this procedure, recoveries of the individual acids from a mixture of volatile fatty acids of known composition added to a washed suspension of LC were within the range of 90–100%.

Reagents. The fatty acids used were purified by distillation using an electrically heated fractionating column packed with Fenske helices and multiturns, and fitted with a reflux head and a device for varying the take-off, and supplied by J. W. Towers and Co. Ltd., Victoria House, Widnes. A high reflux ratio was employed. With this apparatus it was possible to prepare acids which were homogeneous on both the paper and the liquid-gas partition chromatograms. The crystalline sodium pyruvate used was prepared from crystalline pyruvic acid (Elsden & Gilchrist, to be published). The sodium lactate used in the metabolic experiment was prepared from lactic acid, B.P., which is DL, by neutralizing a dilute solution at the boil with N-NaOH.

Gases were freed from O_2 by passage over copper turnings coated with palladium and heated to approx. 400°.

RESULTS

Preliminary manometric experiments with washed suspensions of LC showed that under anaerobic conditions gas was produced from the following substrates: glucose, fructose, sorbitol, mannitol, DL-lactate, pyruvate, DL-serine and formate; but not from DL-alanine, L-aspartate, succinate, acetate, propionate and *n*-butyrate. Experiments in which a carbon dioxide absorber was included in the system showed that both carbon dioxide and a second gas were formed. We assume this second gas to be hydrogen since in our experiments with growing cultures Elsden *et al.* (1951), we analysed the gas formed in the Haldane apparatus and found it to be a mixture of hydrogen and carbon dioxide. Table 1 gives the rate of hydrogen formation (calculated from the linear portions of the progress curves) from a number of substrates. In the case of formate, hydrogen production commenced after a lag period of 30 min. whereas in the case of the remaining compounds the formation of this gas began immediately on adding the substrates. This appeared to indicate that the formic hydrogenlyase enzyme system was adaptive in this organism and that formate is not the precursor of hydrogen. Results

with pyruvate and lactate were peculiar in that there was an initial rapid increase in pressure in the manometers followed by a decrease which suggested to us that gaseous hydrogen was a reactant in the fermentation. The progress curves for hydrogen production from formate, pyruvate and lactate under an atmosphere of nitrogen are given in Fig. 1. In view of these observations we decided to confine our attention to the fermentation of pyruvate.

Table 1. *Rate of hydrogen production from substrates*

(Experiments carried out in double side-bulb cups containing 2 ml. bacterial suspension (0·82 mg. bacterial N), in 0·1 M-phosphate buffer, pH 6·5, containing 0·02% (w/v) Na_2S, $9H_2O$, 20 μmoles of substrate in 0·2 ml. H_2O or 0·2 ml. H_2O in the control added from one side bulb after equilibration. Second side bulb contained 0·2 ml. 20% (w/v) KOH plus filter paper; centre well contained a stick of freshly scraped yellow phosphorus, gas phase N_2; $T = 37°$; $q_{H_2(N)} = \mu$l. H_2/mg. N/hr.)

	$q_{H_2(N)}$		$q_{H_2(N)}$
Control without substrate	30	DL-Serine	119
		L-Aspartate	24
Fructose	385	Succinate	35
Glucose	340	Formate	266
Sorbitol	250	Acetate	34
Mannitol	201	Propionate	38
DL-Lactate	315	*n*-Butyrate	28
Pyruvate	798		

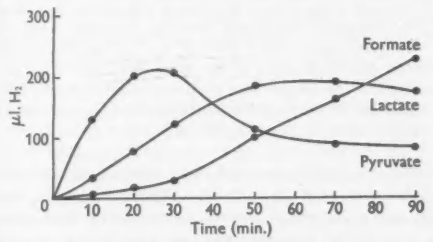

Fig. 1. Rate of production of hydrogen from pyruvate, DL-lactate and formate. Each cup contained 2 ml. bacterial suspension (0·41 mg. bacterial N/ml.) in 0·1 M-phosphate buffer containing 0·02% (w/v) Na_2S, $9H_2O$, pH 6·5; 20 μmoles substrate in 0·2 ml. distilled water placed in side bulb. Centre well contained 0·2 ml. 20% (w/v) KOH. Gas phase N_2; $T = 37°$.

When pyruvate was incubated with a washed suspension of LC under a gas phase of nitrogen the magnitude of the secondary uptake of hydrogen was variable but, when a gas phase of hydrogen was used, it was always marked (Fig. 2). Presumably this was due to a concentration effect. Using Summerson type manometers (Summerson, 1939) fitted with Dixon and Keilin flasks (Dixon & Keilin, 1933), we were able to measure both hydrogen and carbon dioxide production from pyruvate under atmosphere of nitrogen plus 5% carbon dioxide and hydrogen plus 5% carbon dioxide respectively. The results of such an experiment, which was stopped after 60 min., are given in Table 2. It will be seen that, in the presence of hydrogen the amount of

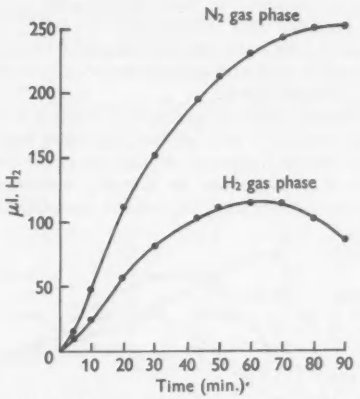

Fig. 2. Effect of nitrogen and hydrogen on production of hydrogen from pyruvate. Each cup contained 0·5 ml. bacterial suspension (prepared by washing and suspending in boiled-out distilled water containing 0·02% (w/v) Na_2S, $9H_2O$, pH adjusted to 6·5 with N-HCl) and 1·0 ml. 0·1 M-phosphate buffer, pH 5·85. 40 μmoles sodium pyruvate in 0·4 ml. distilled water added from side bulb. Centre well contained 0·2 ml. 20% (w/v) KOH plus filter-paper absorber. $T = 37°$.

Table 2. *Production of hydrogen and carbon dioxide from pyruvate*

(Experiments carried out in Summerson manometers with Dixon-Keilin cups which contained 2 ml. bacterial suspension (0·4 mg. bacterial N/ml.) in 0·025 M-$NaHCO_3$ containing 0·02% (w/v) Na_2S, $9H_2O$, cells washed in saline containing 0·9% (w/v) NaCl and 0·02% (w/v) Na_2S, $9H_2O$, pH adjusted to 6·5 with N-HCl.)

	μl. H_2	μl. CO_2	CO_2:H_2
	N_2 + 5% CO_2 gas phase		
(1) Control	11	26	2·4
(2) With pyruvate	101	208	2·0
(2) – (1)	90	182	2·0
	H_2 + 5% CO_2 gas phase		
(1) Control	11	26	2·4
(2) With pyruvate	60	233	3·9
(2) – (1)	49	207	4·2

hydrogen produced is approximately halved whereas the amount of carbon dioxide formed does not differ significantly from that formed under nitrogen. This fits in with the assumption that the organism can utilize gaseous hydrogen during the fermentation of pyruvate.

The effect of pH on the metabolism of hydrogen during the fermentation of pyruvate was next examined. The results are given in Fig. 3 and it will be noted that both output and uptake of hydrogen are most rapid at pH 5·8 and that at pH 7·0, whilst there was an output of hydrogen there was no apparent uptake. In this experiment the cells were washed and suspended in 0·9% (w/v) sodium chloride solution containing 0·02% (w/v) Na_2S, $9H_2O$ and sufficient N-hydrochloric acid to adjust the pH to 6·5.

The most likely interpretation of these observations appeared to us to be that hydrogen was being used to reduce a product or products of the fermentation of pyruvate, and we therefore examined the effect of the addition of acetate, propionate and

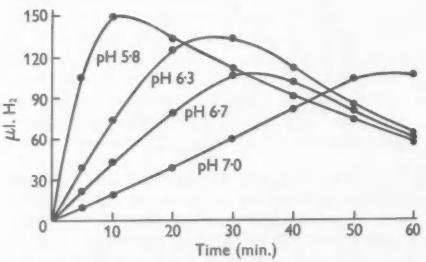

Fig. 3. Effect of pH on production of hydrogen from pyruvate. Each cup contained 1 ml. of bacterial suspension (0·64 mg. bacterial N) prepared by washing and suspending in saline containing 0·9% (w/v) NaCl and 0·02% (w/v) $Na_2S.9H_2O$ and the pH adjusted to 6·5 with N-HCl, and 1 ml. 0·2 M-phosphate buffer of appropriate pH. Centre well contained 0·2 ml. 20% (w/v) KOH plus filter paper, and side bulb contained 15 μmoles sodium pyruvate in 0·15 ml. distilled water. Gas phase N_2; $T = 37°$.

n-butyrate on the consumption of hydrogen during the fermentation of pyruvate. The experiments were carried out under an atmosphere of hydrogen in order to obtain a maximum utilization of this gas. The results of such an experiment are given in Fig. 4. It will be seen that addition of either acetate, propionate or *n*-butyrate results in an increased uptake of hydrogen and that addition of the first two acids resulted in an overall uptake of the gas. At the end of the experiment the contents of each of the cups was acidified and transferred quantitatively to a Markham apparatus and steam distilled. The distillates were titrated with standard (approx. 0·02 N) sodium hydroxide using phenol red as indicator and the neutralized distillates evaporated down almost to dryness; suitable volumes of each of the solutions were then examined by paper chromatography. This showed that, under the conditions of the experiment, acetate and *n*-butyrate were the main products of the fermentation of pyruvate; addition of acetate resulted in an increase in size of the *n*-butyrate spot; addition of propionate caused the appearance of a spot with the same R_F as *n*-pentanoate and addition of *n*-butyrate resulted in the formation of an acid with an R_F the same as that of *n*-hexanoate. In preliminary experiments in which *n*-pentanoate was fermented with pyruvate under hydrogen we observed no increase in hydrogen uptake, nor could we detect an acid corresponding to *n*-heptanoate amongst the end products. The

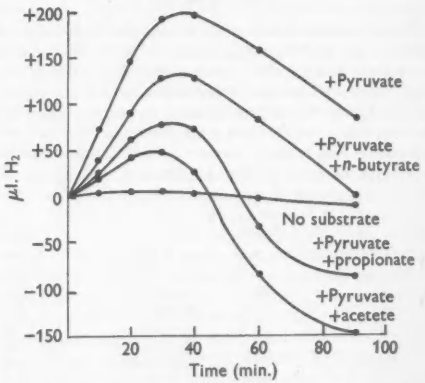

Fig. 4. Effect of acetate, propionate and *n*-butyrate on hydrogen output from pyruvate. Each cup contained 2 ml. bacterial suspension (0·4 mg. bacterial N/ml.) in 0·1 M-phosphate buffer, pH 6·5, and 0·2 ml. 20% (w/v) KOH plus filter paper in the centre well. Side bulb contained 20 μmoles sodium pyruvate substrate in 0·2 ml. water and 20 μmoles of the sodium salt of the appropriate fatty acid. Gas phase H_2; $T = 37°$.

chromatographic method used was, of course, only qualitative, but it sufficed to show that LC synthesizes *n*-butyrate, *n*-pentanoate and *n*-hexanoate in the same general way as was found for *Cl. kluyveri* by Bornstein & Barker (1948).

A quantitative study was next made of these effects. The experiments were carried out in Warburg manometers fitted with double side-bulb cups and the substrates were added from Keilin dangling tubes. Using the procedure described under methods, in conjunction with the liquid-gas partition chromatogram, we were able to estimate all the products of the fermentation in the contents of a single manometer cup. The results of a complete experiment are given in Table 3.

The control without substrate produced relatively large amounts of carbon dioxide and volatile acids and for this reason we give both corrected and uncorrected values for each of the fermentation products. It will be noted that, if the uncorrected values are used, then the carbon recoveries are sub-

Table 3. *Fermentation of pyruvate in the presence of fatty acids*

(Experiments carried out in double side-bulb cups which contained 2 ml. bacterial suspension (0·83 mg. bacterial N/ml.) in 0·1 M-phosphate buffer, pH 6·5. One side bulb contained 0·2 ml. CO_2-free 2 N-NaOH plus a filter-paper absorber; the other, 0·4 ml. 4 N-H_2SO_4. Substrates added from Keilin dangling tube. $T = 38°$, time 90 min. Figures in parenthesis = μmoles fatty acid utilized.)

		With 32·4 μmoles pyruvate		With 32·4 μmoles pyruvate, +27·5 μmoles acetate		With 32·4 μmoles pyruvate, +28·5 μmoles propionate		With 32·4 μmoles pyruvate, +28·5 μmoles *n*-butyrate	
	Control	Observed	Corrected	Observed	Corrected	Observed	Corrected	Observed	Corrected
Hydrogen	−1	+ 1	+ 2	− 8·2	− 7·2	− 6·0	− 5·0	− 2·1	− 1·1
Carbon dioxide	+8·9	+39·3	+30·4	+40·7	+31·8	+41·9	+33	+44·0	+35·1
Acetate	+3·5	+ 7·3	+ 3·8	+ 8·9	+ 5·4 (−22·1)	+ 6·7	+ 3·2	+ 7·6	+ 4·1
Propionate	+2·8	+ 3·1	+ 0·3	+ 4·9	+ 2·1	+10·3	+ 7·5 (−21)	+ 3·6	+ 0·8
n-Butyrate	+1·3	+ 8·9	+ 7·6	+14·1	+12·8	+ 4·7	+ 3·4	+16·2	+14·9 (−13·6)
n-Pentanoate	0	+ 1·5	+ 1·5	+ 4·2	+ 4·2	+14·7	+14·7	+ 3·7	+ 3·7
n-Hexanoate	0	+ 2·3	+ 2·3	+ 4·8	+ 4·8	+ 2·6	+ 2·6	+12·1	+12·1
Residual pyruvate	—	0·3	—	0·1	—	0·4	—	1·0	—
Pyruvate fermented	—	−32·1	—	−32·3	—	−32·0	—	−31·4	—
% C recovery	—	126	94	119	99	108	91	109	94
Oxidation-reduction index	—	—	1·04	—	1·29	—	1·01	—	0·93

stantially above the theoretical, and that the carbon dioxide produced is in excess of 1 mole/mole pyruvate utilized. On the other hand, by correcting for the control values the carbon recoveries are of the order to be expected in this type of experiment; the carbon dioxide/pyruvate ratio approximates to unity and, with the exception of the experiment with acetate the oxidation-reduction ratios (Johnson, Peterson & Fred, 1931) of the fermentations are unity. An oxidation-reduction ratio of 1·0 implies that the oxido-reduction state of the products is the same as that of the substrate or mixture of substrates and hence is a valuable indication of the qualitative and quantitative precision of the analyses. No significance is attached to the discrepancy in the oxidation-reduction index of the pyruvate plus acetate experiment, since a relatively slight error in the estimation of the small amounts of *n*-pentanoate and *n*-hexanoate would be sufficient to account for this.

Table 3 also shows that acetate, propionate and *n*-butyrate are utilized by LC in the presence of pyruvate and hydrogen because the final amounts of these acids, even neglecting the control, are less than the amounts added. Hydrogen and carbon dioxide and the fatty acids containing an even number of carbon atoms are the main products of the fermentation of pyruvate by LC. Addition of acetate resulted in an increased formation of *n*-butyrate. Propionate, as was foreshadowed by the qualitative analyses with the paper chromatogram, increased the yield of *n*-pentanoate; and *n*-butyrate gave rise to *n*-hexanoate.

DISCUSSION

Cl. kluyveri forms fatty acids from ethanol and either acetate, propionate or *n*-butyrate; the oxidation of the ethanol, via acetaldehyde, providing the active acetate necessary for the synthesis. LC utilizes pyruvate in place of ethanol, and we assume that pyruvate acts as the source of the active acetate. Korkes, del Campillo, Gunsalus & Ochoa (1951) and Nisman & Mager (1952) have shown that the oxidation of pyruvate by extracts of *Escherichia coli* and *Streptococcus faecalis* and of *Cl. saccharobutyricum* respectively involves, amongst other things, coenzyme A and a suitable electron acceptor, and they have provided evidence in favour of the view that the acetyl phosphate, which accumulates during the oxidation of pyruvate, arises from a transacetylation reaction between acetylcoenzyme A and orthophosphate catalysed by the enzyme phosphotransacetylase. We have as yet carried out only a few preliminary experiments with cell-free preparations of LC so we cannot say for certain that a similar system is operating in this organism. However, Mr A. C. Warner (unpublished experiments) in our laboratory has made acetone powders of LC which produced hydrogen and carbon dioxide from pyruvate provided that orthophosphate was present; cf. the work of Koepsell, Johnson & Meek (1944) with extracts of *Cl. butylicum*.

The nature of the active acetate involved in the synthesis of fatty acids by *Cl. kluyveri* has not been established with certainty, but it would seem reasonable to suppose that it is acetylcoenzyme A.

The recent work of Stadtman (1952) on the synthesis of acetylcoenzyme A from acetyl phosphate and coenzyme A in the presence of the phosphotransacetylase of *Cl. kluyveri* is in keeping with this view. The organism contains a high concentration of coenzyme A. Dr Mabel Davidson of the Physiology Department, University of Sheffield, kindly assayed freeze-dried preparations of the organism for us, using the method of Kaplan & Lipmann (1948), and found approx. 2000 Lipmann units/g. dry weight which is of the same order as that found for *Cl. kluyveri* (H. A. Barker, private communication).

There appear to be three electron-accepting systems with which the oxidation of pyruvate is coupled, namely, those leading to the formation of molecular hydrogen, of propionate and of *n*-butyrate, *n*-pentanoate and *n*-hexanoate respectively. Assuming that coenzyme A functions in the oxidation of pyruvate by LC, the oxidation and reduction reactions which occur in this organism can be paraphrased as follows with RSH representing coenzyme A:

$$CH_3.C{:}O.COOH + RSH \rightarrow (CH_3CO)SR + 2H^+ + 2e + CO_2, \quad (1)$$

$$2H^+ + 2e \rightarrow H_2, \quad (2)$$

$$CH_3.C{:}O.COOH + 4H^+ + 4e \rightarrow CH_3CH_2COOH + H_2O, \quad (3)$$

$$CH_3.C{:}O.SR + 4H^+ + 4e + \left\{\begin{array}{l} CH_3COOH \rightarrow CH_3(CH_2)_2\,COOH \\ CH_3CH_2COOH \rightarrow CH_3(CH_2)_3\,COOH \\ CH_3(CH_2)_2COOH \rightarrow CH_3(CH_2)_4\,COOH \end{array}\right\} + RSH + H_2O. \quad (4)$$

Equation (1) represents the oxidation reaction and equations (2)–(4), the reduction reactions. Since reaction (4) involves products of reactions (1)–(3) we are of the opinion that it is a secondary reaction. If conditions are such as to favour reaction (2) the end products will consist mainly of the fatty acids containing an even number of carbon atoms, i.e. acetate, *n*-butyrate, and *n*-hexanoate; such a situation occurs when glucose is the substrate. On the other hand, when reaction (3) predominates then the final products will be propionate and *n*-pentanoate and this occurs when lactate is the substrate, Elsden *et al.* (1951).

The only other organism which has so far been found to utilize these three accepting systems is *R. rubrum* (Kohmiller & Gest, 1951). These workers found that the amount of propionate, and hence the amount of *n*-pentanoate, was directly related to the concentration of carbon dioxide in the medium. All we can say on this point is that when LC is grown upon a lactate medium there is only a small fall in the pH at the end of growth and the bound carbon dioxide content is high; but, when glucose is the substrate, the pH falls to approx. 5·0 and the bound carbon dioxide is correspondingly reduced; consequently there may be a correlation between carbon dioxide concentration and propionate and *n*-pentanoate formation. The fact that, under our experimental conditions, LC produced very little propionate from pyruvate (Table 3) might seem to discount this hypothesis; but, since a carbon dioxide absorber was present carbon dioxide tension in the gas phase was negligible. The amount of carbon dioxide which was bound in solution at pH 6·5, which was not measured, would not be very large. That carbon dioxide can enter the catabolic reactions of this organism is apparent from preliminary studies with $^{14}CO_2$. When this is added to washed suspensions fermenting pyruvate, all the acids, but particularly acetate and propionate, contain the isotope. Degradation of the acetate showed that the tracer appeared to be evenly distributed between the methyl and carboxyl carbons. The propionate, on the other hand, was predominantly carboxyl labelled. This could be explained in terms of the formation of succinate by a carbon dioxide fixation reaction followed by a decarboxylation of succinate to propionate as Johns (1951) found in *Veillonella gazogenes*; but here we must point out that LC does not decarboxylate succinate either in washed suspensions or in growing cultures; this, of course, does not necessarily imply that the decarboxylase system is absent, it shows only that the whole organism cannot attack this substance when it is added to the medium.

Finally, it is germane to consider the possible role of LC in the rumen of the sheep. This organism, because of its characteristic morphology, is relatively easy to observe by direct microscopic examination of sheep-rumen contents, and indeed we have so observed it in some, but not all, of our experimental animals. Further, we have always succeeded in isolating it in pure culture from sheep rumen contents. Gray, Pilgrim, Rodda & Weller (1952) and El-Shazly (1952) have found *n*-pentanoate in the rumen contents of sheep; and in addition Gray *et al.* (1952), in *in vitro* studies, have shown that addition of ^{14}C-labelled propionate to rumen contents fermenting wheaten straw results in the formation of labelled *n*-pentanoate, a reaction similar to that which we have observed with washed suspensions of LC. It would therefore seem reasonable to suggest that this organism is, in part at least, responsible for the formation of the higher volatile fatty acids of the sheep-rumen contents. Our experiments were conducted at low tensions of carbon

dioxide whereas, in its natural environment, the rumen of the sheep, the carbon dioxide tension is high, and this must be borne in mind in any assessment of the role it plays in the rumen.

SUMMARY

1. Washed suspensions of LC, a Gram-negative coccus isolated from the rumen of sheep, attack glucose, fructose, sorbitol, mannitol, lactate, pyruvate, DL-serine and formate anaerobically with the production of carbon dioxide and a gas which we assume to be hydrogen.

2. When pyruvate is fermented under an atmosphere of hydrogen there is an initial outburst of hydrogen followed by a consumption of the gas.

3. Addition of either acetate, propionate, or *n*-butyrate to pyruvate fermentations increases the uptake of hydrogen with increased formation of *n*-butyrate, *n*-pentanoate and *n*-hexanoate respectively.

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The Biosynthesis and Metabolism of Betaines in Plants

1. THE ESTIMATION AND DISTRIBUTION OF GLYCINEBETAINE (BETAINE) IN *BETA VULGARIS* L. AND OTHER PLANTS

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Betaine is found in a wide variety of plants (Guggenheim, 1940) and occurs in relatively high concentration in the tissues of members of the Chenopodiaceae and the Gramineae and also in *Lycium chinense* Mill. (Solanaceae), and *Vicia* species of the Leguminosae. Betaine occurs in all organs of these plants, the highest values usually being found in the leaves. In animal tissues betaine is formed by the enzymic oxidation of choline (Mann & Quastel, 1937) and plays an important part in the process of transmethylation (Muntz, 1950). It was therefore of interest to study the biosynthesis and metabolism of this compound and of other betaines in the plants in which they normally occur.

Since betaine is a very stable substance, methods available for its estimation have been restricted to the use of non-specific precipitants such as potassium triiodide (Staněk, 1904; Blood & Cranfield, 1936; Reifer, 1941), phosphotungstic acid (Davies & Dowden, 1936) and ammonium reineckate (Walker & Erlandsen, 1951). For the purposes of the present

investigation an accurate and reasonably rapid method for the estimation of betaine in plant tissues was desirable and since none of the above methods proved reliable when applied to young actively growing tissues, attention was directed to the modification and improvement of the reineckate method of Walker & Erlandsen (1951). This method is based upon the fact that betaine, being a very weak base, forms with strong acids salts which can be titrated with alkali. Walker & Erlandsen (1951), in the application of their method to beet molasses and diffusion juices, first precipitated betaine as the reineckate which was filtered off and dissolved in aqueous acetone. The reineckate ion was then removed with silver nitrate and the betaine nitrate thus formed was titrated with alkali to pH 6 with methyl red as indicator. The authors state that although choline and other basic nitrogenous substances are precipitated by ammonium reineckate, the salts formed on treatment of the precipitate with silver nitrate are not sufficiently acidic to be titrated with alkali to pH 6.

When this method was applied to purified extracts of tissues of young roots and seedlings it became apparent that other basic substances were present which were coprecipitated with betaine when the extracts were treated with ammonium reineckate at pH 1. The reineckates of some of these substances gave acidic salts when the reineckate ion was removed with silver nitrate and were thus included in the estimation as betaine. It was found that most of the interfering substances could be effectively removed by treatment of the plant extracts with silver oxide according to the procedure of Herbst & Clarke (1934), who showed that amino acids and partially methylated amino acids, but not betaine, undergo oxidative deamination when boiled with silver oxide.

EXPERIMENTAL AND RESULTS

The estimation of betaine in plant tissues

The plant material was dried at 80° in an oven with a forced draught, finely powdered, and 0·1–1·0 g. was weighed into the extraction thimble of a micro-Soxhlet apparatus and extracted with methanol for 1 hr. The extract was transferred to a boiling tube, small pieces of porous pot were added to prevent bumping and the methanol removed at 80° on a water bath. The residue was taken up with distilled water (5 ml.) and powdered activated charcoal (0·5–2 g.) was added according to the amount of plant material originally weighed out. The solution was brought almost to boiling and filtered through a sintered-glass crucible, the disk of which was covered with a thin layer of Gooch asbestos. The colourless filtrate was collected in a test tube and the boiling tube rinsed three times with 2 ml. portions of hot water which were run through the crucible and added to the filtrate which was then transferred quantitatively to a small evaporating dish and concentrated on a boiling-water bath to approx. 3 ml. The concentrated solution was returned to the test tube and the basin washed twice with 1 ml. portions of water giving a total volume of 5 ml. The pH of the solution was adjusted to 1 (approx.) by addition of 0·05 ml. of 10 N-HCl, and 10 ml. of a saturated solution of ammonium reineckate (previously adjusted to pH 1) were added and the tube kept at 4° for 1 hr.

The reineckate precipitate was filtered off in a chilled sintered-glass crucible of medium porosity and washed four times with 2 ml. portions of water-saturated ether. The underside of the crucible was dried and the precipitate dissolved in 75% (v/v) acetone (5 ml.). The reineckate ion was next removed by the addition of 5 ml. of $AgNO_3$ reagent (a solution 0·1 N with respect to both $AgNO_3$ and $NaNO_3$), the precipitate of silver reineckate centrifuged off and the clear supernatant decanted into a boiling tube. The precipitate was washed twice with 5 ml. portions of $AgNO_3$ reagent and twice with 5 ml. portions of distilled water, and the washings added to the boiling tube together with small pieces of porous pot. The boiling tube was warmed gently to remove acetone and 4 ml. of 0·2 N-NaOH were slowly added and the solution boiled gently until the volume was reduced to 5 ml. (approx.). After cooling, 0·15 ml. 10 N-HCl was added and the precipitate of AgCl removed by filtration through a sintered-glass crucible, the disk of which was covered with Gooch asbestos. The AgCl precipitate was washed three times with 2 ml. portions of water and the combined filtrate and washings were concentrated to 3 ml. (approx.) in a small basin on the water bath and returned to the test tube. The basin was washed twice with 1 ml. portions of water and the washings added to the test tube to make a total volume of 5 ml. (approx.). The pH was adjusted to 1, 10 ml. of saturated ammonium reineckate solution were added and the tube kept at 4° for 1 hr. The precipitate was filtered off in a chilled sintered-glass crucible of medium porosity and washed with 3 ml. portions of water-saturated ether until the washings were acid-free. The betaine reineckate was dissolved in 75% acetone (v/v) and the solution collected in a conical centrifuge tube of 10 ml. capacity. Next 5 ml. of $AgNO_3$ reagent were added and the silver reineckate removed by centrifugation. The supernatant was carefully decanted off into a 100 ml. conical flask and the silver reineckate was washed free of betaine nitrate with 5 ml. portions of distilled water to a total volume of 40 ml. (filtrate + washings). To the combined filtrate and washings were added 4 drops of 0·1% methyl red dissolved in ethanol and the solution was titrated to pH 6 with 0·02 N-diphenylguanidine in 70% ethanol: 1 m-equiv. of betaine nitrate = 1 m-equiv. of diphenylguanidine. (The stock solution of 0·05 N-diphenylguanidine in 70% ethanol was prepared from diphenylguanidine twice recrystallized from boiling toluene (Carlton, 1922).)

It will be observed in the procedure for removal of interfering substances that Ag_2O is produced from the excess of $AgNO_3$ remaining after removal of the reineckate ion as silver reineckate. The amount of NaOH added is carefully adjusted so that a slight excess of unchanged $AgNO_3$ remains in the solution. The pH of the solution is thus maintained at a point near neutrality, a condition essential for complete oxidation of amino acids and partially methylated amino acids (Herbst & Clarke, 1934). Guanine and adenine, if present, are coprecipitated with betaine but are removed as insoluble silver compounds when the crude reineckate precipitate is dissolved in aqueous acetone and treated with $AgNO_3$. Other betaines such as trigonelline, stachydrine

and betonicine are unaffected by treatment with Ag_2O and if present, will give relatively insoluble precipitates with ammonium reineckate at pH 1. On removal of the reineckate ion with silver nitrate, the nitrates of these betaines will give acidic solutions and will be estimated as betaine. Caution is therefore necessary in the interpretation of results if the presence of other betaines has been established by preliminary examination of tissue extracts by means of paper chromatography: the melting point and chromium content of the purified betaine reineckate were therefore used as criteria of purity. The method gives the most satisfactory results when the amount of betaine in the sample of plant material taken for analysis lies within the range 1–10 mg. A recovery of 99–101 % was obtained when known amounts of betaine were added to tissue extracts and to mixtures of amino acids and purines.

Application of the method to the determination of betaine in Beta vulgaris *L., and other plants*

As soon as possible after harvesting, the plant material was dried and stored in a desiccator until required for analysis. Except where otherwise stated, all leaves (with petioles) of the shoot system of individual plants were included in the sample taken for analysis. Table 1 shows the values for betaine (mean values of two estimations) in plants of *Beta vulgaris* L., *Atriplex patula* L., and *A. hortensis* L., in progressive stages of development. In Table 2 the values for betaine in various other plants are given.

DISCUSSION

Staněk (1906, 1911), as the result of an investigation of the betaine content of a variety of plants, concluded that young actively growing tissues show a higher concentration than mature tissues. The results of the present investigation substantially support the findings of Staněk with the exception that leaves of young seedling plants showed a lower betaine content than the leaves of older plants. In fact, the betaine content of the whole leaf system of young plants increased throughout the vegetative

Table 1. *The betaine content of tissues of* Beta vulgaris *L.*, Atriplex patula *L. and* Atriplex hortensis *L. during the growth period*

Plant	Age (days)	Betaine content (g./100 g. dry wt.) Leaf	Petiole	Root
Beta vulgaris	25	0·25	—	—
	33	0·36	—	1·27
	44	0·48	—	—
	56	0·64	—	—
	62	0·84	—	1·15
	75	0·87	—	—
	81	1·02	—	—
	96	1·32	1·09	—
	132	1·86	1·65	0·79
	140	2·20	—	—
	167	2·55 (outer leaves) 3·34 (middle leaves) 4·85 (inner leaves)	1·08 (upper half) 0·99 (lower half)	0·18
Atriplex patula	30	1·32	—	—
	60	1·71	—	—
	80	2·64 (plants in bud)	—	—
	90	2·48 (plants in flower)	—	—
	120	1·84 (plants fruiting)	—	—
A. hortensis	39	0·78	—	—
	48	1·04	—	—
	107	0·99 (old leaves) 1·26 (young leaves)	—	—

Table 2. *The betaine content of miscellaneous plants*

Plant	Material	Betaine content (g./100 g. dry wt.)
Spinacea oleracea L.	Leaves (35 days)	0·27
Chenopodium vulvaria L.	Leaves (mature)	1·86
C. bonus-henricus L.	Leaves (mature)	2·23
Atriplex canescens James	Leaves (mature)	3·27
A. halimus L.	Leaves (mature)	4·90
Amaranthus caudatus L.	Leaves (mature)	0·54
Lycium chinense Mill.	Leaves (mature)	1·95
Vicia faba L. Seedlings germinated in dark	Cotyledons	0·22
	Shoots	1·47
	Roots	1·10
Wheat (*Triticum vulgare* Vill. var. Atle) Etiolated seedlings (8 days)	Coleoptiles	0·83
	Roots	0·42
Beta vulgaris L. Etiolated leaves from mature roots	—	5·00

period and reached a maximum at the time of flowering. Moreover, young seedling leaves of *Beta vulgaris* contained much less betaine than young actively growing leaves sprouting from the crown of a mature root. A concentration gradient of betaine is established during the vegetative phase of growth of the plants examined, the betaine content rising steadily from the root to the leaf. On the contrary, the young unfolding leaves at the centre of the crown of the mature root contain more betaine than the older leaves at the periphery of the crown. Leaves of the shrubby evergreen species of Atriplex (*A. halimus* and *A. canescens*) are noteworthy for their high content of betaine. Care is necessary in the interpretation of results of the analysis of young as compared with mature tissues when dry weight is taken as the basis of calculation.

However, an explanation of the above results is possible in the light of recent work which has been carried out on the formation of betaine in *Beta vulgaris* (Cromwell & Rennie, 1953). This work has shown that betaine is formed in the root tissues by the enzymic oxidation of choline. Since choline oxidase is not present in the leaf tissues it seems likely that betaine must have been translocated from the roots to the leaves. Failing the utilization of betaine in metabolic processes, it would be expected that, with continuous production in the root, a gradual accumulation of betaine would take place in the shoot system during the growing period. Similarly, the high concentration of betaine in the young leaves growing from the centre of the crown of a mature root could be explained as due to translocation from a relatively large mass of root tissue which in the resting condition contains a considerable amount of betaine. The high concentration of betaine in the leaves of the perennial evergreen species likewise could be explained as the result of a slow accumulation over a period of time.

SUMMARY

1. A method for the estimation of betaine in plant tissues is described.

2. This method incorporates an oxidation procedure for the removal of substances which are present in young tissues and which interfere in the estimation of betaine by the reineckate method.

3. The improved method has been employed for the estimation of betaine in organs of various plants in all stages of development, and the conclusion is reached that betaine formed in the roots is translocated to the shoot system and accumulates in the stems and leaves.

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